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The incorporation of carbon 14 into leaf amino acids and protein

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THE INCORPORATION OF CARBON¹⁴ INTO
LEAF AMINO ACIDS AND PROTEIN

by

David W. Racusen

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Plant Physiology

Approved:

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1953

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INTRODUCTION

General Problems

The synthesis and fate of the protein molecule in the living organism is a matter of outstanding importance to the physiologist and biochemist. Reasons for this are easily found when one considers that proteins are (1) catalysts of bio-systems, (2) the basic heredity unit when combined with nucleic acids, and (3) the material of which viruses are composed (again in combination with nucleic acids). In addition to these major roles, one might also point out the possible significance of proteins in the buffering of cytoplasmic solutions, and their effect on the passage of electrolytes through membranes.

A wide and varied literature has accumulated concerning protein and amino acid metabolism. Very little is known, however, concerning the synthesis and fate of proteins and amino acids in green plants.

One of the phenomena most readily demonstrated in green leaves is the rapid decline in protein nitrogen following excision of the leaf. In the past, it has not been possible to determine whether this protein loss was due to a lowered rate of protein synthesis or an increased rate of protein hydrolysis (14). Either hypothesis is possible, assuming that leaf proteins, like the studied animal

proteins (38), are normally undergoing simultaneous synthesis and degradation. However, this fundamental fact has not been confirmed in plants.

If the excision phenomenon is a reflection of the leaf's dependence on the root, then it is likely that the leaf's synthetic abilities are in some way limited. Thus, a leaf may be dependent on special products of root synthesis. The comparison of excised and attached leaves, on the basis of synthetic abilities or limitations, might provide clues as to the special functions of the individual organs (i.e., leaves and roots).

Another obscure feature of plant nitrogen metabolism is the synthesis of "essential" amino acids. Green plants are apparently unique in their ability to synthesize all amino acids from CO_2 and NH_3 . The origin of the aromatic ring in phenylalanine, etc., and the branched chain in leucine, etc., seems especially interesting in that these are structures requiring a mode of synthesis not fully understood in any biological system. However, in attempting to elucidate these mechanisms in higher plants, one must be certain first, of the synthetic sites. An assumption that these syntheses occur in the leaf may not be true.

The possibility that leaves may be dependent on special products of root synthesis was noted in connection with the excision phenomenon. Assuming now that the loss of protein is due to a lowered rate of protein synthesis, it is conceivable that roots supply some precursor essential for normal synthesis. The required

precursor could be an "essential" amino acid. If this is true, an excised leaf could be expected to synthesize little, if any, of the particular amino acid.

The obscurity concerning "essential" amino acid does not extend to all amino acids. Previous workers (47) have shown that both alanine and serine arise rather directly from the primary photosynthate (glyceric acid or etc.), which is, in turn, formed by condensation of CO_2 with a two-carbon unit. The early appearance of C^{14} -labeled aspartate (10) from C^{14}O_2 would likewise indicate its origin from malic acid (or oxalacetate), following the condensation of pyruvate with C^{14}O_2 . The origin of glycine is somewhat more mysterious, in that this compound is uniformly labeled in short time (47).

In addition to the lack of information on essential amino acids, no investigation has been made on the incorporation of particular amino acids into leaf protein. This is a difficult problem to approach for two reasons: (1) leaf cells cannot be conveniently exposed to labeled substrates other than C^{14}O_2 (see p. 17), and (2) the isolation of individual leaf proteins, on a micro-scale, has not yet been accomplished. This latter fact can best be appreciated when one considers the primitive state of knowledge existing in the composition of leaf proteins.

Protein Composition of Leaves

Protein molecules may differ from each other, not only in size, amino acid composition, and sequence, but also in molecular shape. Thus, the separation of a given species from other proteins is often a difficult matter. This is particularly true of plant proteins.

On the basis of separability, leaf proteins have been considered of two types; non-soluble and soluble. The non-soluble proteins are found associated with particulate structures; nuclei, chloroplasts, and mitochondria. Such protein accounts for up to 45% of the total leaf protein nitrogen (51). The soluble fraction is present in the protoplasmic ground substance of the cytoplasm. A comparison of chloroplasts and soluble leaf proteins shows that they are very similar in amino acid composition (9).

Great difficulty has been encountered in solubilizing chloroplast protein. In two instances, at least, a green protein solution has been obtained which is electrophoretically homogeneous, and has a molecular weight of about 5×10^6 . However, there is some possibility that the observed homogeneity is an artifact (51).

The most completely characterized unit has been the soluble protein. It is known, for example, that this consists of two main fractions which are separable both by electrophoresis and ultracentrifugation. Results based on the latter method have also shown that the portion termed "Fraction I" (41) protein is apparently a

single nucleoprotein species, which constitutes 23 to 50% of the total cytoplasmic protein in dicotyledonous plants. The "Fraction II" (51) protein is a mixture of uncertain composition.

Protein Turnover in Leaves

It is a well known fact that almost all biological molecules are in a constant state of flux. This synthesis and breakdown of metabolites has its significance in the fact that all molecules having a truly dynamic role, as opposed to mere storage function, must suffer chemical change in the course of action.

When one considers the dynamic role played by certain proteins (i.e., enzymes, antibodies, etc.), it seems reasonable that such action should be accompanied by some change in the protein molecule. However, it is also true that the more passive plasma proteins, and other proteins of the animal body, are in a constant state of flux (38).

Historically speaking, this was not an obvious fact. If the concentration of a particular metabolite remained constant, there was indeed no way of guessing that the life of any particular molecule in the pool had a definite duration. This situation can best be described as a "steady state"; the rate of synthesis equals the rate of degradation, so that there is no change in the concentration of the substance. The use of tracers makes it possible to measure this "turnover". "Turnover time" is a way of expressing the results of such measurement. This may be defined as the time required to

completely replace all the molecules in a particular pool. Theoretically, the measurement of turnover time is quite simple. For example, the carbon-14 labeled precursor (A), of a particular metabolite (B), may be administered to a test organism. At timed intervals, a sample of the metabolite (B) is withdrawn and assayed for radioactivity. This information may then be used in calculating the rate at which the labeled precursor (A), is converted to (B). If (B) is in a "steady state", it follows that its rate of disappearance must equal the rate at which (A) is converted to (B). By knowing the amount of (B) in the organism, it is thus possible to determine how long it would take to replace completely a pool of this size.

In practice, such measurements are often very difficult to obtain. The measurement of leaf protein turnover time is especially arduous.

By feeding $C^{14}O_2$ to an illuminated leaf, the leaf protein will rapidly become radioactive. The rate of C^{14} entry into the protein may be measured as a function of time. Such measurements would, by themselves, be useless for purposes of determining turnover time. Several reasons make this true.

It is well known that leaves do not normally exist in a relative steady state (in contrast to adult mammals). Leaves are either growing or approaching senescence. It would be difficult to state that, at any particular time during their lives, the excretion of waste nitrogenous products equalled the rate of protein synthesis. The

incorporation of C^{14} into protein would therefore be of doubtful significance, and might only indicate a net synthesis of protein. However, even if it were known that the C^{14} incorporation occurred during a period in which there was no change in the total amount of protein, the significance of such "turnover" would still be uncertain. The following considerations make this obvious.

The fact that proteins are composed of many amino acids makes it likely that different portions of the same protein molecule are turning over at different rates. One might expect that terminal, or otherwise peripheral amino acid residues, would "turnover" more rapidly than the interior residues. The mechanism of peripheral turnover might be a simple exchange between the free and bound amino acid. It is thus reasonable that the significance of turnover may depend upon the exact location of turnover in the protein molecule.

The term, "leaf protein", is rather meaningless. Many different proteins are contained in the leaf. It is inconceivable that every protein molecule in the leaf would turn over at the same rate.

For these reasons, a meaningful picture of leaf protein turnover would necessitate (1) the isolation and assay of individual amino acids from known locations in particular proteins, and (2) an assurance that this protein was in a steady state during the experimental period. The aforementioned state of leaf protein chemistry makes this task very difficult.

The work of Vickery, et al.(50) has shown that the feeding of N^{15} results in an incorporation of this isotope into plant protein, to the extent that 10% of the protein nitrogen had originated during the experiment (72 hours). This result has been interpreted to mean that protein hydrolysis is compensated for by a corresponding protein synthesis. In a general way, this appears to be true. However, this work and its interpretation is somewhat ambiguous.

The indeterminate nature of this finding resides in the fact that the total protein was studied rather than a single protein species. For this reason, a different interpretation may be given the experimental facts; namely, that incorporation of N^{15} may be taking place to form a type of protein molecule differing from that molecule which is being hydrolysed. This would further imply that the protein character of the leaf would be undergoing change with age. There are some existing data to show that this may be a valid conclusion. Pearsall (31, 32) has found that the ratio of "basic N" to "total N" (as determined by Kjeldahl method, etc.) decreased with the age of the leaf. This finding has led Pearsall to make the interesting speculation that there may be two general types of protein; one arising during growth, and another arising from photosynthesis in the mature leaf.

In a recent paper by McCoy, Sublett, and Dobbs (28), an attempt was made to investigate the relation between the amino acid content and the development of the oat plant. For this purpose the plants were

grown under different light periods to produce a graduated series of specimens having the same chronological but different physiological ages. At the end of the experiment the leaves, etc. were harvested and analysed for the total content of the seventeen common amino acids. The results were complex, but showed that relatively large changes in the proportions of the various amino acids did occur with age. Unfortunately, the experiment was not designed to measure the protein amino acids and "soluble" amino acids (i.e., the amino acids extractable by 80% ethanol prior to hydrolysis) separately. However, the changes in amino acid composition do point out the fact that changes in protein composition with age are plausible.

Another facet of leaf protein change with age is the rather exaggerated one of leaf excision.

Leaves, which are detached from the parent plant, with their petioles in water, suffer a loss of protein which is proportional to time (14). Axelrod and Jagendorf (4) have found that during a period when a 50% reduction in total protein had occurred, the content of several enzymes remained unchanged. To maintain this steady state in the presence of general protein loss could imply that leaves preferentially synthesize certain proteins, depending on the prevailing environmental or metabolic conditions.

Purpose

An examination was made of (1) amino acid and protein metabolism of excised and attached leaves, (2) the relative turnover rates of free

amino acids, (3) the origin of leucine and phenylalanine, (4) amino acid and protein synthesis of leaves in the dark, and (5) amino acid and protein synthesis in immature leaves. In addition to the above observations, an attempt was made to (1) determine the turnover rates of individual leaf proteins, and (2) prepare a tissue-free leaf cell suspension, which may be used as a system for the study of protein and amino acid metabolism.

GENERAL EXPERIMENTAL METHODS

In essence, a rather typical experiment would contain the following sequence of events:

- (1.) feeding the leaf $C^{14}O_2$ or other labeled substrate,
- (2.) killing the leaf by immersion in boiling 80% ethanol or liquid nitrogen,
- (3.) extraction of 80% ethanol-soluble materials,
- (4.) hydrolysis of 80% ethanol-insoluble materials,
- (5.) isolation and quantitation of metabolites following extraction and hydrolysis via;
 - (a.) ion exchange, and
 - (b.) chromatography, and
- (6.) degradation of labeled molecules.

In addition to the above, two less general techniques have been resorted to, viz.;

- (1.) the preparation of cell suspensions from soybean leaf, and
- (2.) the isolation of leaf proteins.

Feeding Methods

General

Feeding a leaf $C^{14}O_2$ is, in many instances, the most satisfactory type of carbon-14 administration. The utilization of CO_2 is not only very rapid, but also the starting material ($BaC^{14}O_3$) is considerably more economical than any other C^{14} -labeled substrate. Unfortunately, $C^{14}O_2$ is not a "specific" tracer (except, of course, in a

photosynthetic sense). For those cases where one wishes to investigate a single metabolic path (e.g., glycine to serine), a specific tracer (e.g., glycine-2- C^{14}) is necessary.

In almost all of the present work, $C^{14}O_2$ was used as the tracer. This means, of course, that those materials which are most closely related to members of the photosynthetic cycle will be the most rapidly labeled. Compounds whose synthesis might require some pre-formed carbon structure, which is in turn made by the root, would be labeled at a rate possibly limited by the rate of translocation. By considerations such as these, plus other data, it was hoped that $C^{14}O_2$ feeding would illuminate the major paths of amino acid and protein metabolism.

Feeding chambers

Three types of $C^{14}O_2$ feeding chambers were used. For the purpose of exposing an attached leaf to $C^{14}O_2$, the apparatus described by Vernon (48) was convenient. In brief, this chamber consisted of two glass hemispheres which were fitted together at ground flanges. The leaf petiole ran through a clay-packed indentation in the flanges in such a manner as to expose only the single leaf to the $C^{14}O_2$ (Fig. 1). A finger pump (Sigmamotor, Inc.) served to keep the gas in circulation.

For purposes of feeding excised leaves, a plastic chamber with hinged lid generally was used (Fig. 1 and 2). The small internal volume of the chamber allowed for increased pickup of $C^{14}O_2$, without the necessity of using a circulating pump. At the experiment's

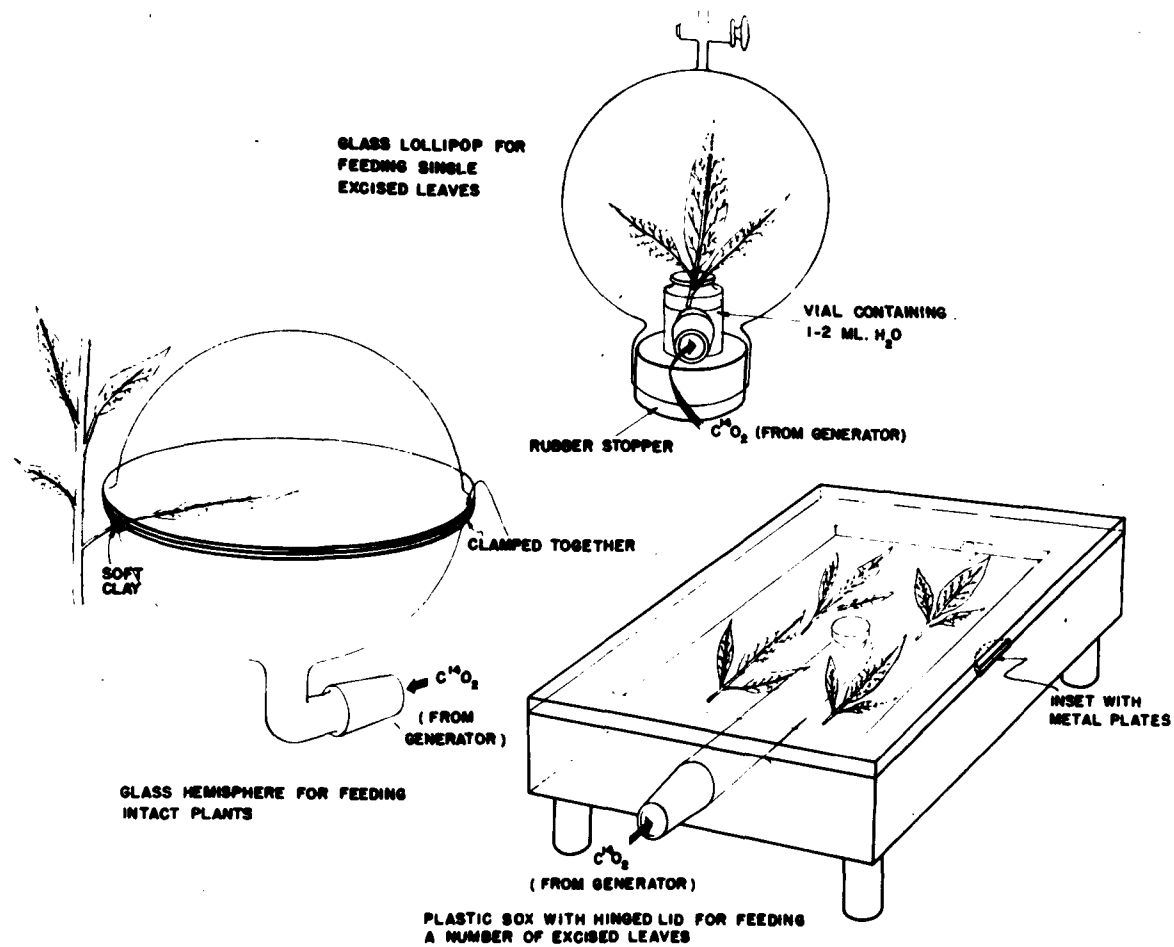
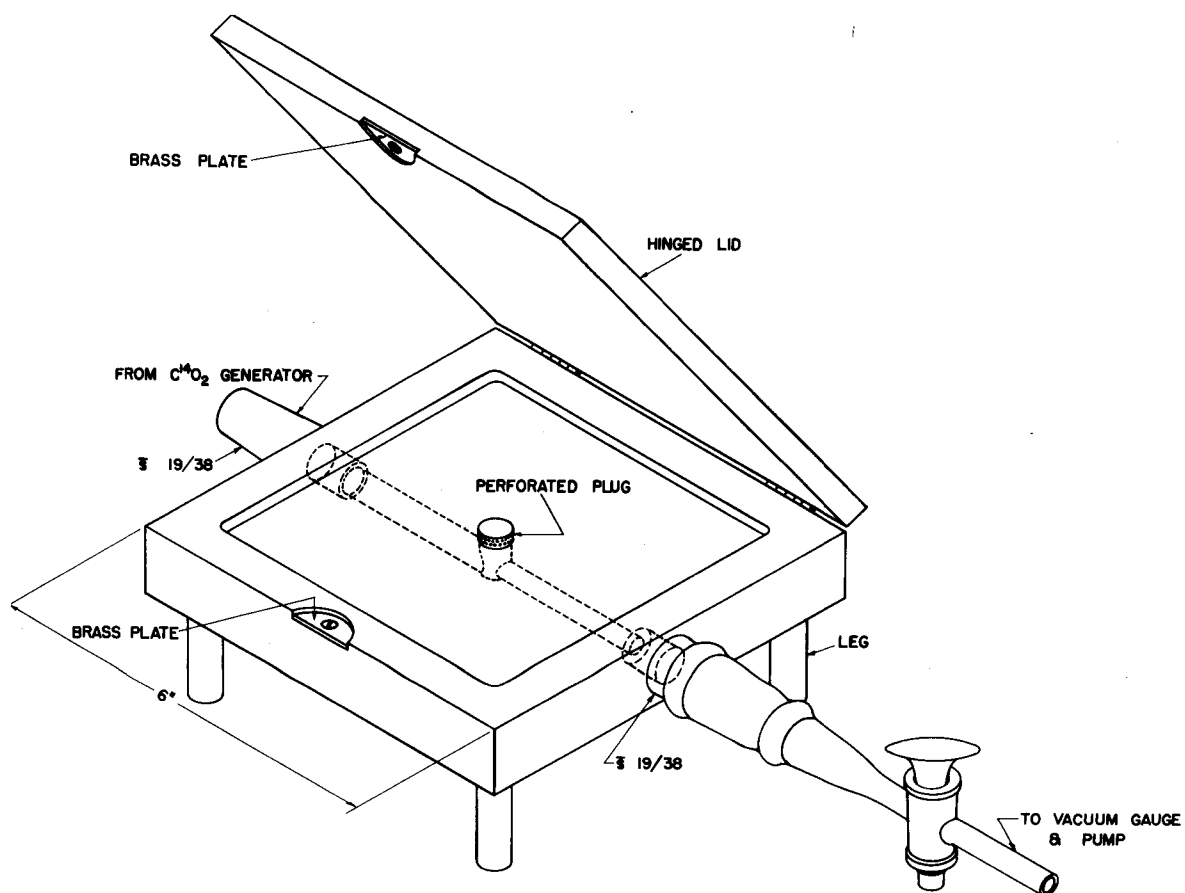


Fig. 1. $C^{14}O_2$ Feeding Chambers.



VOLUME OF CHAMBER ca. 75 ml.

Fig. 2. Plastic C¹⁴O₂ Feeding Chamber.

termination, the lid was pryed open by insertion of a screw driver between the brass plates. In order to move $C^{14}O_2$ from the generator to the chamber, a partial vacuum (about 12 inches of mercury) was applied to the chamber before the generator stopcock was opened. A liberal application of Celvacene (light) to the lid edges made the apparatus air tight.

It was necessary to supply water to the petiole when exposing leaves to $C^{14}O_2$ for long time periods. This was done by feeding in a "lollipop" chamber (Fig. 1).

$C^{14}O_2$ generator

The apparatus which has finally evolved in this laboratory seems much superior to earlier model generators. It has the virtues of a small internal volume, and of being less subject to accidental generation of $C^{14}O_2$. As shown in Figure 3, the apparatus was fabricated from a large, hollow stopcock. A small vacuum (about 5 inches of mercury) was pulled on the generator and the $C^{14}O_2$ generated by the injection of 5-10% perchloric or phosphoric acid. When thus generated, the $C^{14}O_2$ was conveniently stored until the time of feeding.

Preparation of $NaHC^{14}O_3$

A 0.1 M solution of $NaHC^{14}O_3$ was prepared in the following manner.

$BaC^{14}O_3$ (9.4 mg.; 7.2 millicuries/millimole) was carefully placed to the center well of a conical, side-armed Warburg vessel (about 25 ml.); 0.10 M NaOH (0.50 ml.) was placed in the center well and the

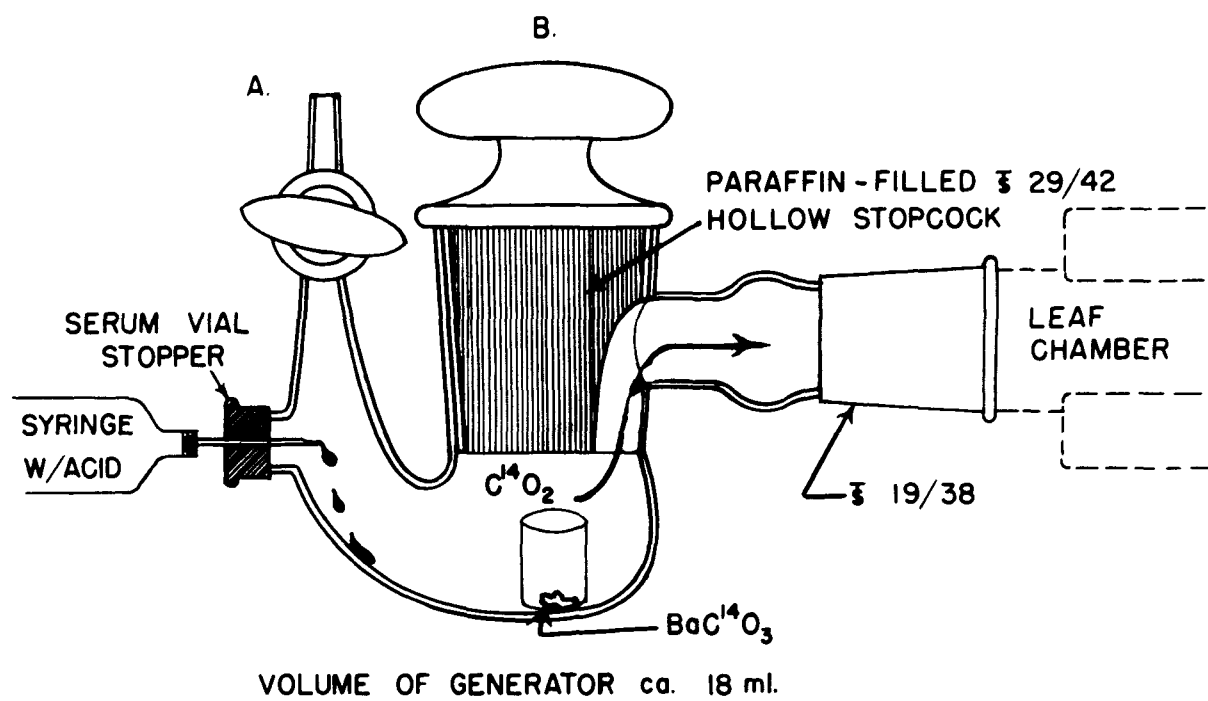


Fig. 3. $C^{14}O_2$ Generator.

side-arm was filled with 5% H_3PO_4 (Fig. 4). After replacing all stoppers tightly, the acid was carefully tipped into the flask. An "incubation" time of 12 hours insured the quantitative formation of $\text{NaHC}^{14}\text{O}_3$. In the event of long storage (1 week, etc.), the vessel should be stored in the cold. Upon prolonged standing the H_2O in the $\text{NaHC}^{14}\text{O}_3$ solution will diffuse into the more concentrated H_3PO_4 leaving a solution of unknown concentration. This situation could be corrected by making the fugacities of both solutions equal.

This solution was useful for the administration of C^{14}O_2 to cell suspensions.

Glycine-2- C^{14} administration

The difficulty of administering a C^{14} -labeled substrate, other than C^{14}O_2 , to leaves is a major stumbling block in metabolic investigations. Three general methods are available : (1) translocation through the petiole, (2) vacuum infiltration, and (3) leaf spraying (24). Only one has been used in the present work; translocation of a solution through the petiole into the leaf blade. This technique requires that sufficient time be given for the solute to reach the metabolic reaction site. Obviously, a uniform concentration of solute will not surround each cell of the leaf. Studies on the rate of substrate utilization and turnover are thus not feasible by this means, with the possible exception of those cases wherein the utilization and turnover are much slower than the rate of solute entry into the leaf. However, this method is useful for qualitative studies on the fate of a particular metabolite.

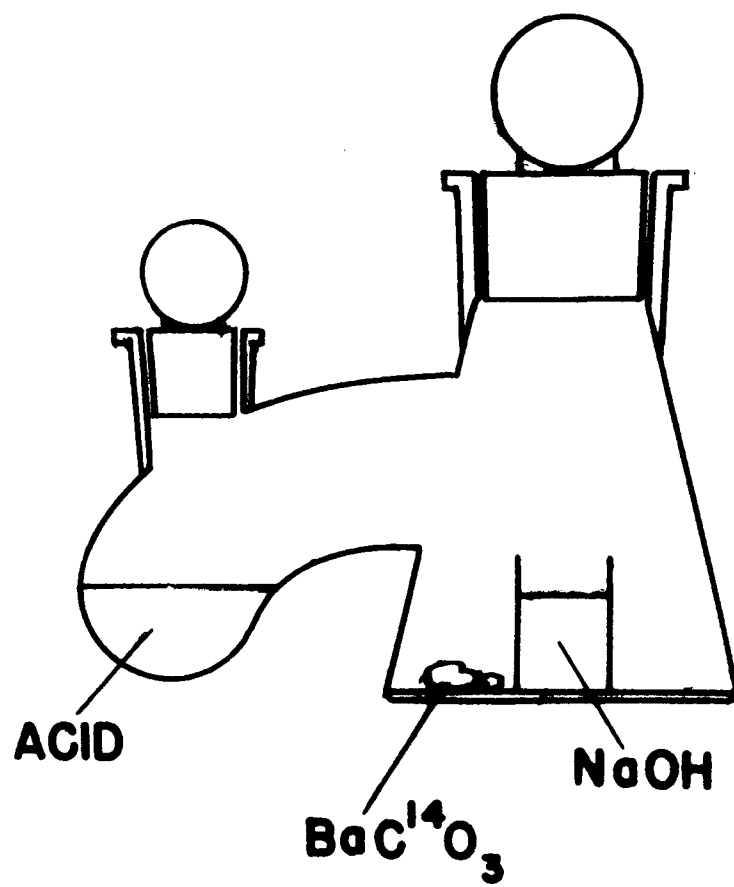


Fig. 4. Preparation of $\text{NaHC}^{14}\text{O}_3$.

The leaf was removed from the parent plant by making a rapid, slantwise cut on the petiole. Excision was performed under a surface of water with a sharp razor blade. The petiole of the detached leaf was kept in tap water for several hours prior to the experiment. During this period, the leaf was subjected to the same light and temperature conditions which were to be used during feeding. By such careful pre-treatment, it was hoped to minimize wounding, allow for a steady state to be attained, and to determine whether or not the leaf would be viable^a under the experimental conditions.

Glycine-2-C¹⁴ (1-2 mg.; 1 millicurie/millimole) was placed in a small glass vessel (inside diameter about 6 mm.) and dissolved in 100 microliters of H₂O (or an amount sufficient to barely cover the cut end of the petiole). The leaf was carefully observed after transfer to this solution, water was added when the cut section threatened to be exposed to air. The experiment was terminated by immersing the leaf in boiling 80% ethanol.

^aExcision of leaves is not always successful. Injury to vascular elements of the petiole may occasionally slow water movement to the extent that wilting occurs. In such an event, movement into the leaf ceases, and the experiment is vitiated.

Light source

In all leaf feeding experiments, an unfrosted, 150 W. incandescent bulb was used as the light source. The filament of the bulb was at a distance of one foot from the leaf surface and, at this distance, delivered a light intensity of 275 foot candles.

Monitoring

It was occasionally useful to determine the uptake of $C^{14}O_2$ during the course of an experiment. For this purpose, the simplest expedient was the insertion of a thin window G. M. tube into the circulating radioactive atmosphere. Commercial models of a suitably modified G. M. tube were available but were unreliable. After contact with $C^{14}O_2$, the tube window became excessively contaminated, yielding a background count of 173 counts per minute (normal background is about 30 counts per minute). This $C^{14}O_2$ pickup was apparently irreversible, as determined by passage of inert 5% CO_2 over the window with no diminution of the activity.

A more reliable piece of apparatus may be constructed from a tube whose window has not been coated with black paint (24).

Critique on $C^{14}O_2$ administration

In general, there are two principal ways of carrying out an isotope experiment; steady state and transient state administration of the labeled substance.

The more widely used method consists of feeding the label for a very short time period as compared to the experiment length (i.e., transient state feeding). This allows one to gather data concerning rate processes and the determination of precursor relationships (36).

The less common method involves feeding the label at a constant rate for the duration of the experiment (i.e., steady state feeding). This method also is useful in establishing precursor relationships.

Both methods have certain difficulties. In transient state feeding it is difficult to supply sufficient label for the determinations of metabolite specific activities. Steady state feeding, while supplying material of high specific activity, is a very exacting method.

In most of the reported work, neither of these two critical methods were applied. The reasons for not doing so were the aforementioned difficulties in either obtaining sufficiently high levels of activity or maintaining a given level of $C^{14}O_2$. In addition, there was apparently little to be gained; the needs of the investigation combined with the lack of specificity of $C^{14}O_2$ made the more rigorous methods superfluous.

There is, however, one possible serious objection to the simpler feeding method. As was indicated previously, the feeding apparatus is a closed system. At the beginning of an experiment, the CO_2 concentration was calculated to be about ten times that of the normal

atmosphere (when using the plastic chamber and 2 mg. of BaCO_3). After one hour the CO_2 concentration was less than that of the atmosphere.

The lack of a constant CO_2 pressure can be expected to alter the rate of processes directly dependent on CO_2 concentration (i.e., carboxylations and decarboxylations). It was assumed that, once formed, the amino acids would be metabolized at a rate reasonably independent of CO_2 pressure. However, in one instance, short time feeding was applied. This was the experiment dealing with the rate of amino acid turnover in leaf punches (p. 72).

Extraction and Hydrolysis

Extraction with 80% ethanol

By means of boiling 80% ethanol it is possible to make a useful separation of leaf components. Furthermore, this reagent is an excellent killing agent so that no transfer is necessary between the two stages; killing and extraction.

With one such extraction (50 ml. of 80% ethanol for 30 minutes with about 400 mg. of fresh tissue), it was possible to remove 90% of the ethanol-extractible material. As a general course, at least two extractions were made prior to hydrolysis of the insoluble residue. This remaining residue consisted chiefly of cellulose, starch, protein, and lignin.

Quantitative extraction was of basic importance. The appearance of amino acid activity in protein may have several different meanings.

Such activity could indicate either physical adsorption or incorporation. (The incorporation could further be due to either simple exchange at peripheral locations or to actual turnover, as indicated on p. 7). The very fact of amino acid incorporation into protein may be questioned if the extraction were less than quantitative. This is a question considered by Simpson and Tarver (40) in their investigation of amino acid incorporation by liver slices. These workers showed that the amino acid incorporation into liver protein was not due to physical adsorption by the demonstration that there was no uptake at 0° C., anaerobically, or in the presence of respiratory poisons.

In addition to these criteria, other workers (39) have shown that the incorporation of the radioactive L-amino acid into protein could not be duplicated by the D-isomer. Furthermore, none of the incorporated amino acid could be removed by incubation with large amounts of inert carrier, hot trichloroacetic acid, alkaline washing, ninhydrin, or oxidation with performic acid.

In the present work, it has been possible also to show that amino acid incorporation is a reality and not due to incomplete extraction. Two experiments served to demonstrate this.

In one case, the dark fixation of $C^{14}O_2$ by a leaf (p.92), it was possible to detect a much different ratio of protein radioactivity to free amino acid radioactivity, when compared to $C^{14}O_2$ pickup in the light. In another instance, a leaf cell suspension contained no labeling in the protein in the presence of highly labeled free amino

acids (p.83). These examples served to confirm the reality of amino acid incorporation.

Alcoholic extraction may be far from quantitative when using systems other than leaves or intact cells. A case in point was the "incorporation" of glycine-2-C¹⁴ into the protein of a chloroplast preparation. Repeated extractions of the acetone precipitated material continued to yield activity after five treatments. The adsorption was probably caused by the presence of free starch grains and finely divided chloroplast fragments.

Extraction apparatus and method

Leaves were most simply killed by rapid immersion in a beaker containing 50 ml. of boiling 80% ethanol. Extraction was allowed to proceed for 30 minutes at a moderate reflux rate. A globe-type water condenser allowed one to reflux the alcohol solution in the beaker without loss of solvent. During extraction, the fleshy portions of the midrib and petiole were crushed with a stirring rod.

An apparently reliable gauge of complete extraction was the total removal of chlorophyll from all parts of the leaf.

The lipid fraction (chlorophylls, carotenes, fats, etc.) was removed by extraction of the 80% ethanol extract with an equal volume of Skelly A. This purification was helpful in preparing good chromatograms of the total extract.

Prior to ion exchange and chromatography, it was usually necessary to concentrate the solution. This operation was performed in

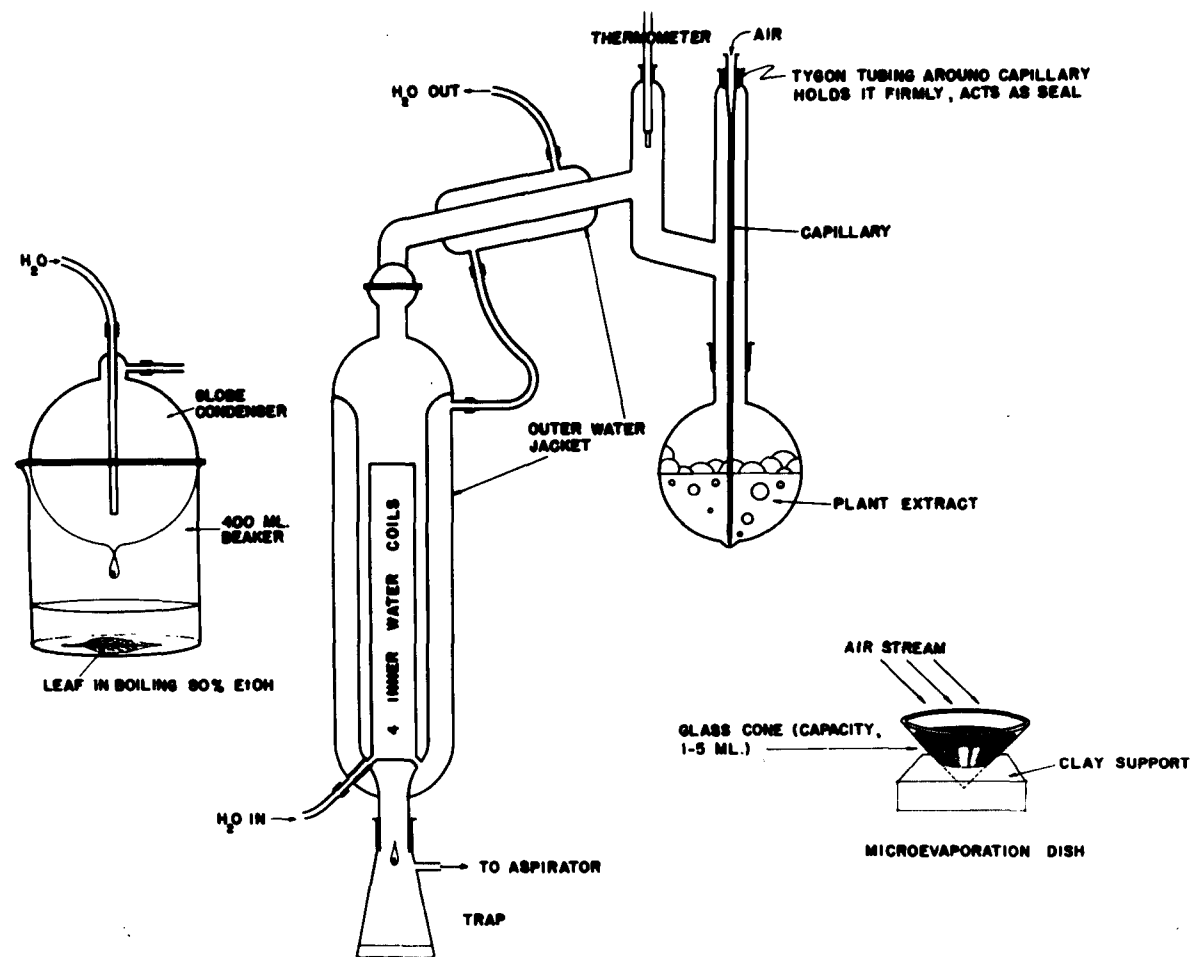


Fig. 5. Extraction and Concentration.

two stages, depending on the amount of concentration which was needed. For purposes of ion exchange, it was necessary to reduce the volume to approximately 5 ml. The apparatus used (Fig. 5) allowed one to rapidly concentrate the ethanol solution to a few milliliters at essentially room temperature. An ordinary aspirator produced sufficient vacuum for this purpose. Further concentration in this apparatus was difficult in that superheating and loss of the material on the flask walls affected both the quality and quantity of recovered material. These objections were overcome by use of the micro evaporating dish, depicted in Figure 5. It was thus possible to concentrate 100 ml. of radioactive extract to 50 microliters, in essentially 100% yield. Application of these 50 microliters to a paper chromatogram was quickly performed. The extra manipulation more than compensated for the time loss and annoyance accompanying the repeated application of a dilute solution.

Hydrolysis of the 80% ethanol-insoluble residue

The direct hydrolysis of the alcohol insoluble residue in boiling 6 N HCl resulted in the formation of brown or black pigments (humins) within two or three hours. After 12 hours of such treatment, approximately 85% of the activity in the starch was lost. The loss of protein radioactivity was not measured, but it was assumed that certain of the amino acids had been at least partially destroyed. That such destruction can be a source of error in the amino acid analysis of proteins is evidenced by the immense literature on this subject (7).

Humin formation appears to be a function of carbohydrate concentration (7). The reaction is catalysed by trace mineral contaminants in hydrochloric acid (27).

Constant boiling hydrochloric acid could have been used in the present work. However, it was felt that the trace minerals remaining in the leaf, even after exhaustive ethanol extraction, would make such a refinement futile.

By taking advantage of the following two facts it was possible to reduce humin formation:

- (1.) Humin formation is proportional to carbohydrate concentration.
- (2.) The hydrolysis of starch is essentially complete after three hours at 100 ° C. in 1 N HCl.

The method used throughout the reported work is described below.

The alcohol-extracted leaf was placed in a 12 ml. centrifuge tube and hydrolysed for 24 hours at 75 ° C. with 5 ml. of 1 N HCl. This treatment was most conveniently carried out by closing the tube with a small marble and placing in a drying oven at 75 ° C. At the end of hydrolysis, the remaining leaf tissue was centrifuged down and the clear supernate removed. In a typical experiment this supernate contained approximately 85% of the carbohydrate activity and approximately 50% of the protein activity. Analysis of the resultant solution by paper chromatography and ion exchange showed that almost 95% of the amino acid activity was in the form of free amino acids. The remaining 5% did not correspond to known amino acids and may have been

peptide units. Continued hydrolysis of the solution in 6 N HCl did not reveal the presence of any new amino acids but only the slight reenforcement of those already noted. In the ensuing studies, it was felt that continued hydrolysis of the 1 N HCl solution was unnecessary.

The remaining leaf residue was suspended in 6 N HCl and hydrolysed, as above, for another 24 hours. The soluble and insoluble residue (termed "crude fiber") were separated by filtration through a small filter paper disk. The "crude fiber" was washed with water and dried with a current of warm air on the filter paper. When thoroughly dry, the crude fiber sample was counted on the paper without further treatment.

Under normal operating conditions, the 1 N HCl hydrolysate was stored until the following day. At this time it was combined with the 6 N HCl hydrolysate and the entire mixture was vacuum distilled to remove HCl and finally ion exchanged.

The method described has certain obvious advantages over the more rapid sealed-tube and reflux methods. This was especially true when a number of samples were run simultaneously. In the present work the extra time required for hydrolysis was used to complete separations on the ethanol-soluble materials.

Ion Exchange

The use of ion exchange had but one object; the removal of contaminants which would interfere with paper chromatographic separations. These contaminants were; (1) the ammonium chloride and metallic

chlorides formed during hydrolysis of the leaf, and (2) the radioactive sugars and acids normally formed during photosynthesis. The latter materials were most easily removed by passage through a bed of Amberlite IR-120 in the H^+ cycle. The cationic materials (all amino acids,^a ammonium ions, and metallic ions) are held by the resin. The effluent contains the neutral (sugars, etc.) and anionic (organic acids, etc.) materials. The complete removal of these materials was important for the reason that in most instances they constituted the major portion (about 80%) of the radioactivity and thus could cloak the position of possible amino acids on the chromatogram.

The interference created by ammonium and metal chlorides was of a different nature. In this case, the contaminants brought about irregularities in the size, shape, and location of the amino acid spots. A number of investigators have remedied this situation by "de-salting", prior to chromatography, by either electrical (17) or ion exchange methods. The method finally used was a modification of that of Carsten (13). This method relies on the fact that ampholytes, bound to a resin in the acid cycle, may be removed with NH_4OH . Materials of stronger cationic character, i.e., most metal ions, would remain bound to the resin. With this means it is possible to separate the amino acids from mineral contaminants.

^awith the possible exceptions of taurine and cysteic acid

In the present method, a small column of IR-120 was used. Previous work had shown that ion exchange resins may retain some radioactivity (possibly 5% of the total) after passage of a radioactive solution through the column. For this reason new columns were prepared for each use.

This was most easily done by having on hand a supply of previously charged and washed IR-120. The charged, clean resin was stored under water in a flask and for use was simply transferred to the column with the aid of a wide mouth pipet.

Small columns were used (Fig. 6) in order to minimize the volume of eluent and the time necessary for elution. The bed volume used was approximately 8 mm. x 150 mm.

The radioactive extract, in 5 ml. of H_2O , was placed on the column. After this volume of material has been absorbed into the bed, a small amount of water (approximately 5 ml.) was added to wash the upper part of the column. Water was added until 25 ml. of effluent had been collected. This solution was assayed by plating a known volume on a glass disk.

Elution of the amino acids was accomplished by running 25 ml. of 3 N NH_4OH through the column. Assay was performed on glass as above. The solution was concentrated in vacuo prior to chromatography.

When used as described, the method gave a salt free solution of amino acids suitable for chromatography. The elution of amino acids was quantitative, except for arginine, lysine, and histidine, as

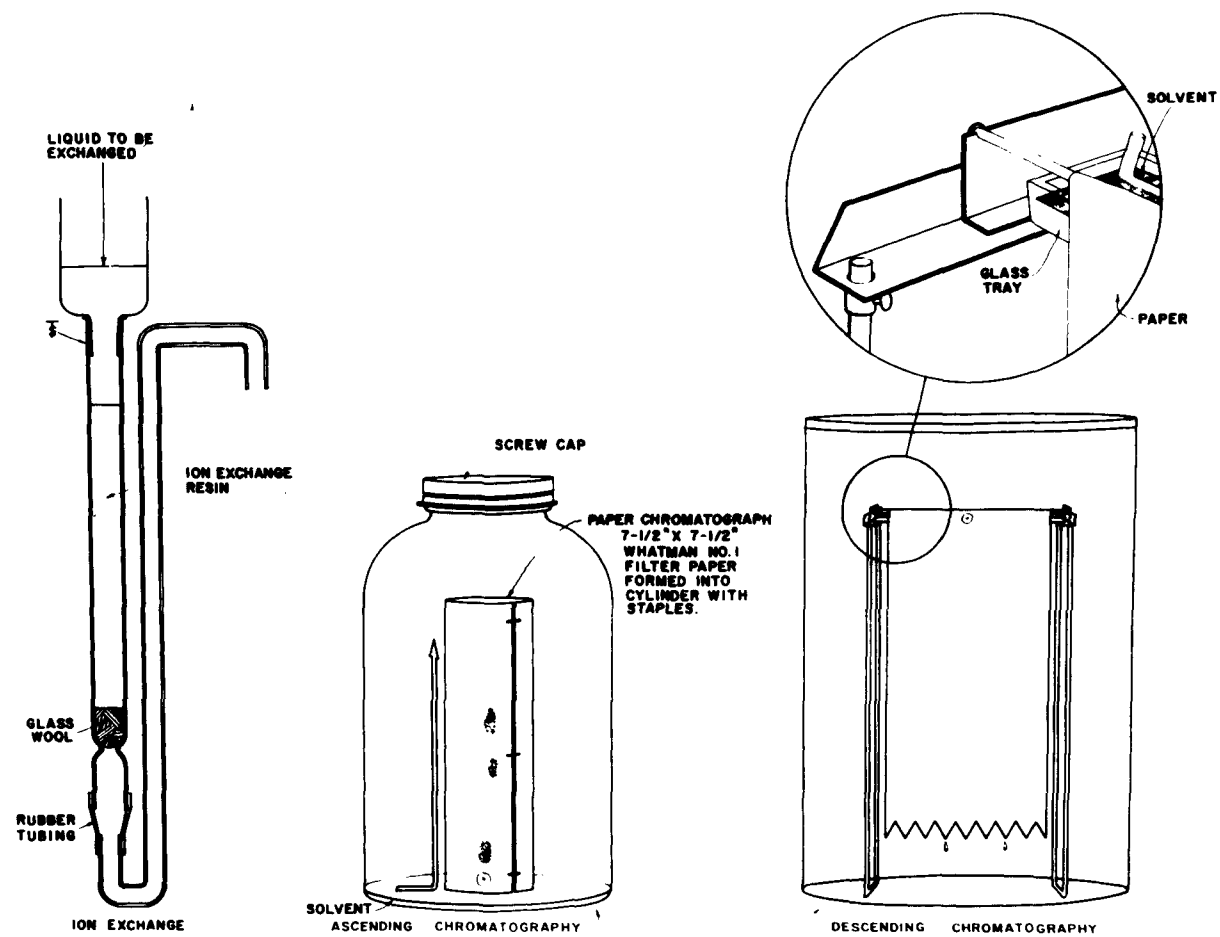


Fig. 6. Ion Exchange and Paper Chromatography.

determined by quantitative paper chromatography. The recovery of arginine and lysine was approximately 57% and that of histidine somewhat higher (approximately 65%). In all of the reported data, figures for these compounds have been suitably corrected.

Elution from IR-100, as performed by Carsten (13), was apparently quantitative for all amino acids. It was found, however, that this resin gave a certain amount of color "throw" (i.e., some resin was dissolved by the NH_4OH). When placed on paper this contamination caused difficulties similar to that produced by mineral contaminants, thus nullifying the previous purification.

Paper Chromatography

Quantitative method

The technique of quantitative analysis by means of filter paper chromatography has been reviewed by Block, Le Strange, and Zweig (8). These authors noted the following general methods:

- (1.) Visual comparison of spot size and color intensity with a standard
- (2.) Elution of material with subsequent measurement of color, etc.
- (3.) Area of spot
- (4.) Total color of spot
- (5.) Maximum color density
- (6.) Area x density method
- (7.) Retention analysis

Of these various methods, the "Area of Spot" technique was chosen for use because of its simplicity and accuracy, about 5% (8).

In essence this method is as follows:

The mixture of amino acids is placed at the origin and distributed over the filter paper by solvent flow. After spraying with ninhydrin and heating, the colored spots are carefully marked and the areas measured. Each area will be proportional to the logarithm of the concentration of each amino acid (19). Although simple in principle, certain precautions must be observed in order to obtain adequate precision.

- (1.) It was observed that spot areas of identical concentration were not identical when obtained under even slightly different conditions (areas are greater at higher temperatures and with longer runs on the paper). To obtain meaningful data, it is necessary to make simultaneous comparisons with a standard solution on the same sheet of paper.
- (2.) Only spots which are reasonably symmetrical and well separated from other materials may be used.
- (3.) A number of replicates on the same sample must be run. The results of such comparisons should be within the desired per cent variation. (In some instances, an error of 5% in area measurement may lead to a 10% error in the final result).

The above requirements restrict one to the use of one-dimensional passes even though the two-dimensional method allows for better

resolution. In order to separate all amino acids, a number of different solvents were used in long one-dimensional passes on replicate samples.

Irregular movement of spots (i.e., streaming, fronts, etc.) was best controlled by the use of relatively pure amino acid solutions and buffered paper and solvent. The means by which this was done is discussed in detail.

- (4.) Non-uniformity of paper is a possible source of error, especially in cases where the buffered paper is used, and the spot area is measured by weighing the cut-out paper. In twenty five trials, the maximum variation of buffered paper weights, of the same area, was $\pm 4.3\%$.

The method of McFarren (29) was especially valuable in the separation of amino acids in one dimension. By the use of long descending passes, it was found possible to separate the twenty common amino acids by running replicate samples with both paper and solvent buffered at a selected pH.

In the present work, it was necessary to use only two buffered solvents and papers in order to separate most of the amino acids commonly occurring in soybean and tobacco leaves. These were (in order of excellence): (1) phenol and paper saturated with pH 12.0 phosphate buffer, and (2) m-cresol and paper saturated with pH 8.4 borate buffer.

Occasionally, it was useful to allow the phenol solvent to drip off the serrated ends of the paper in order to improve the separation

of asparagine and glycine^a (Fig. 6). When this was done, a small aliquot of the dye "Fast Green" ($R_f = 0.41$) was run next to the amino acid mixture in order to visualize the optimum time of solvent flow. In the case of m-cresol, "Tropaeolin 000" was found a convenient marker ($R_f = 0.58$).

The amino acids were detected with a ninhydrin solution of the following composition: 0.4% ninhydrin dissolved in 50 parts of H₂O saturated phenol, 50 parts of 95% ethanol, and 10 parts of glacial acetic acid. To develop the colors, the paper was heated at 75° C. for 5 minutes. This reagent was sufficiently acid to give an optimum pH for the ninhydrin reaction on the buffered paper.

The fact that one cannot pre-calibrate the method by drawing curves from experimental data, forces one to calculate each concentration separately. Certain mathematical considerations make these calculations possible when a minimum of three spots are applied to a single chromatogram. The following derivation and description demonstrates this fact.

^aIt was found that this system gave a poor separation of glutamine and alanine. To correct this situation, a portion of the extract was hydrolysed in 1 N HCl prior to chromatography. When this was done, the asparagine and glutamine were converted to the free acids and the "drip" method was not required.

If the logarithm of concentration is proportional to the spot area, then it follows that a change in concentration (C) produces a proportional change in the spot area (A),

viz: $\Delta \log C$ proportional to ΔA

therefore, $d \log C = k dA$,

and $\int d \log C = k \int dA$ (equation 1).

Integrating without limits one obtains:

$$\log C + a = k(A + b) = kA + kb,$$

$$\log C = kA + kb + a.$$

If one adds the constants (kb, and a) to yield a new constant (B),

$$kb + a = B,$$

one obtains the equation (the equation of a straight line with intercept B),

$$\log C = kA + B \quad (\text{equation 2}).$$

By integrating equation 1 between limits one obtains:

$$\int_{C_2}^{C_1} d\log C = k \int_{A_2}^{A_1} dA$$

$$\log C_1/C_2 = k(A_1 - A_2).$$

Let C_1 and C_2 be different sized aliquots of the same solution, placed on different spots of the same paper. After chromatography, the respective areas will be A_1 and A_2 .

If $C_1 = 2C_2$, then the expression becomes (solving for k),

$$k = \frac{0.30}{(A_1 - A_2)}.$$

It is thus possible to calculate k from two spots of unknown concentration if the ratios of their concentration are known.

In practice, twice as many 5 microliter aliquots were placed on one of the two spots of unknown composition^a. After chromatography and ninhydrin treatment, the spot areas were marked, cut out, and weighed to the nearest 0.1 mg. The constant, k , was then calculated from the difference in weights, $A_1 - A_2$ ^b.

^a 5 microliter pipets were obtained from Microchemical Specialties, Berkeley, California.

^b spot area is proportional to spot weight

On still another spot was placed a sample of known composition (concentration = C_3 , area = A_3). After measuring this spot, it was possible to calculate B from k, obtained above, and equation 2, i. e.,

$$B = \log C_3 - kA_3.$$

The value for B was now used in equation 2, to determine the concentration of the "unknown" spot.

Alternatively, the data were substituted in the equation (derived from the preceeding simpler equations):

$$\log C_1 = 0.30 \frac{(A_1 - A_3)}{(A_1 - A_2)} + \log C_3.$$

By this method it was possible to determine 20 micrograms of an amino acid with an accuracy of approximately 10%.

Qualitative method

In order to identify all the amino acids in a leaf extract, it is necessary to prepare only a single two-dimensional chromatogram. This desirable circumstance is due to the work of Levy and Chung (27), which was based on the method of McFarren (29).

In brief, this method utilizes long descending passes (about 18 inches) in first, an acidic solvent (BAW: n-butanol-acetic acid-water; 4:1:5 by volume, the organic phase was used) and secondly, with a solvent (m-cresol-phenol; 1:1 by volume, and then saturated with the borate buffer) and paper buffered with borate at pH 9.3. Following

completion of the first pass, the paper was removed from the chromatography chamber and dried. The one dimensionally spread amino acids were covered by two narrow glass strips (2 inches wide) and the paper was sprayed with the borate buffer. When dry, the glass strips were removed and the chromatogram was run in the *m*-cresol-phenol solvent.

The amino acids were detected with a ninhydrin reagent of the following composition: 0.1% ninhydrin in 95% ethanol containing 5% collidine. (On the buffered paper used, this reagent was preferable to the preparation on p. 35.)

A faster, simpler method of amino acid identification is that reported by Redfield (37). Although the separations are not as well defined as those obtained by the above method, the procedure is very useful. The Redfield technique is based upon the use of very small two-dimensional sheets (13.75 cm. x 14.50 cm.) of Schleicher and Schuell No. 507 filter paper, which are run ascendingly (Fig. 6). For present use, sheets of Whatman No. 1 ($7\frac{1}{2}$ inches x $7\frac{1}{2}$ inches) gave satisfactory results.

In the first dimension, a mixture of methanol-water-pyridine (80:20:4, by volume) was used. The second dimension was run in a mixture of tert.-butanol-methyl ethyl ketone-water-diethyl amine (40:40:20:4, by volume). The spot containing the amino acid mixture was neutralized by exposure to ammonia vapor prior to chromatography. It was particularly vital to have salt free amino acid solutions when using this system.

In some cases, small two-dimensional papergrams were run in n-butanol, n-butyric acid, and water (2:2:1, by volume) and 80% phenol (80 phenol:20 water, by volume). This system gives a poor separation of glycine, serine, and glutamic acid (Fig.10).

The amino acids have also been run by means of paper electrophoresis in order to isolate the basic amino acids. Contrary to its usual behavior, arginine forms a very discrete spot when run in this manner (Fig.11).

Radioautography

The completed paper chromatograms were exposed to Kodak No-Screen X-Ray Film for a suitable time period. An exposure of 24 hours was generally sufficient for autography of a chromatogram whose origin contained 300 x 64 counts per minute (counted on paper with a G. M. tube of approximately 7% efficiency). More or less time is required depending upon the number of compounds into which the activity must be divided.

After development of the radiogram, it may be accurately superimposed on the chromatogram. This was done by matching the images of radioactive ink spots with the corresponding actual spots placed on the paper prior to autography (48). Such alignment allows one to outline the radioactive areas with a pencil. The penciled areas may then be counted, directly on the chromatogram (48), prior to ninhydrin treatment. Much of the reported data was collected in this manner.

Identification

Most identifications were performed by means of paper chromatography. The exact correspondence of radioactivity with the ninhydrin reacting carrier, in different solvents, was generally sufficient for identification. In doubtful cases, the radioactivity was eluted from the paper, and co-crystallized with its possible, non-radioactive counterpart. Identity was assumed when during several recrystallizations, no change in specific activity occurred.

Counting

Radioactive samples were generally counted to within a 3% statistical error. Samples placed on glass disks, or spread on filter paper disks, were counted in either Q-Gas, or with a Geiger tube, depending upon their activity and the need for accuracy. Most often, a Geiger tube was satisfactory. The type used was the Nuclear Instrument and Chemical Corp. Model D-34, having a window thickness of 1.4 mg./cm.² and an efficiency of approximately 7%. For counting with this device, a specially built plate holder was necessary (Fig. 7).

Degradation of Labeled Molecules

The most satisfying evidence, with reference to studies on biosynthetic pathways, is that obtained via degradation. The means by which this is done are exceedingly diverse and depend, not only on the particular molecule, but also, on the information which is desired.

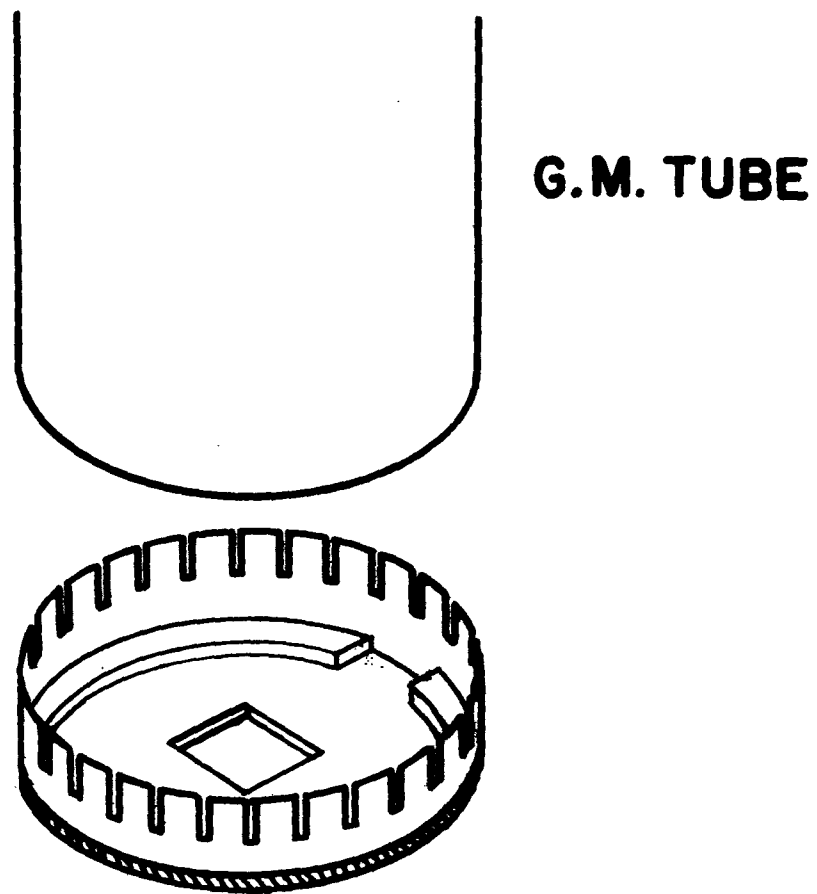
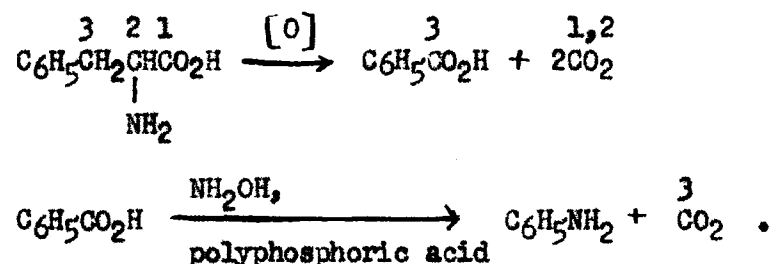


Fig. 7. G. M. Tube Plate Holder.

In the present work, three different amino acids were degraded; phenylalanine, arginine, and leucine. The degradations were not complete in the sense of each carbon atom of the chain being assayed. Degradations were carried only to the extent of determining the specific activity of certain significant carbons.

The degradation of phenylalanine

The primary purpose for degrading phenylalanine was to determine the amount of radioactivity in the aromatic nucleus as compared to the side chain. The overall scheme was as follows:



With two exceptions (the decarboxylation of benzoic acid and the isolation of aniline), the method finally used was that evolved by Gilvarg (21). This involved side chain oxidation with sulfuric acid and potassium dichromate.

The spot identified as phenylalanine was eluted from the paper chromatogram with 2 ml. of water, and co-crystallized with 100 mg. of inert carrier by the addition of 2 ml. of 95% ethanol. Recrystallization from 50% ethanol yielded a product of radiochemical purity (yield 70%). A portion was oxidized to CO₂ (11,46) and counted as BaCO₃.

Fifty mg. of the above phenylalanine, one g. of potassium dichromate, and 3 ml. of 25% sulfuric acid (by weight) were placed in a 30 ml. round bottom flask. A sharp, glass chip was added and the mixture was refluxed at 130°C . for 5 hours^a. It was important to use a straight walled, water cooled condenser to insure adequate return of the volatile intermediates (phenylacetaldehyde and its polymers). After cooling to room temperature, the reaction mixture was twice extracted with benzene. The benzene extract was concentrated to near dryness, in a porcelain evaporating dish, on a steam bath. On complete evaporation of the benzene, benzoic acid was deposited on the surface of the evaporating dish (yield 40%). The crude benzoic acid was recrystallized from hot water. A portion (approximately 3 mg.) of the pure material was converted to CO_2 (11, 46) and counted as BaCO_3 .

The further degradation of benzoic acid to aniline was performed by the method of Synder, et al. (42) rather than that of Gilvarg (21). This latter method consisted of a Schmidt degradation, via sodium azide, followed by conversion of the aniline to acetanilide. In the present work, it was found that this method was laborious and gave poor yields (maximum yield, 27%).

^aThe evolved CO_2 was occasionally assayed. In all such cases, the activity was too low, thus indicating that some ring destruction had occurred. These results have not been reported.

The method which was found most convenient was based upon a relatively new reagent, polyphosphoric acid^a.

In a typical experiment, 50 mg. of benzoic acid, 35 mg. of hydroxylamine hydrochloride, and approximately 500 mg. of polyphosphoric acid^b were placed in a reaction vessel. A spiral bubbler containing CO₂-free 0.1 N NaOH was connected to the system (Fig. 8). By means of an aspirator, and Ascarite containing tower, CO₂-free air was pulled through the reaction mixture and bubbler.

The temperature of the flask was raised to 160° C. At the end of twenty minutes, the reaction was complete and the oil bath removed. The spiral bubbler was removed and the CO₂ was precipitated and counted in the form of BaCO₃ (yield 40%). The assay of this material represented carbon 3. After cooling to room temperature, the reaction mixture was cautiously made basic (pH 10) by the dropwise addition of 50% sodium hydroxide. The aniline was transferred to benzene by making two equal volume extractions of the cooled, basic solution. The aniline-benzene solution was dried by allowing it to remain in contact with solid potassium hydroxide for 30 minutes.

^a A very generous sample was supplied by the Victor Chem. Works.

^b Pouring this viscous material was normally difficult. It was therefore useful to pre-heat the container in hot water in order to lower the viscosity prior to transferring.

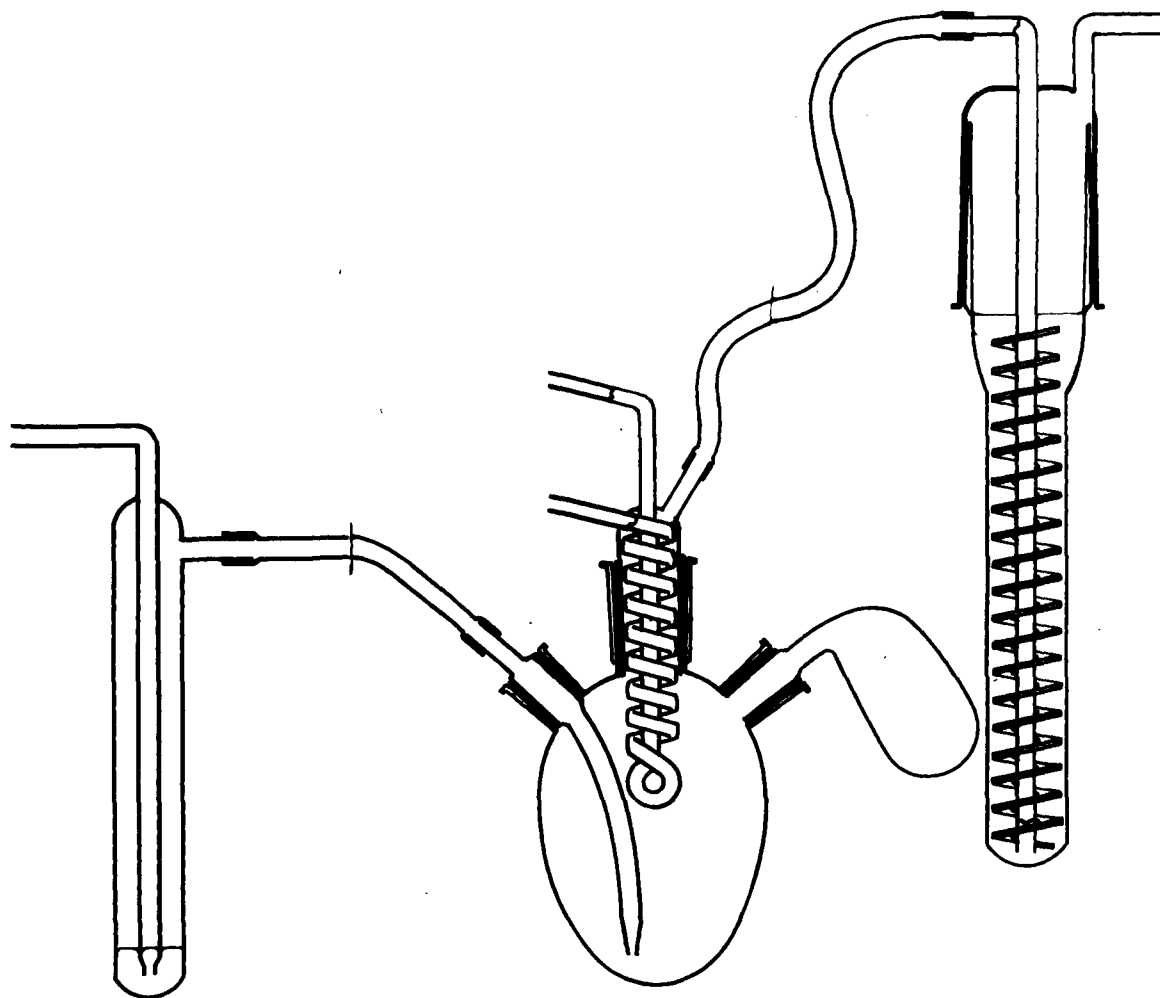


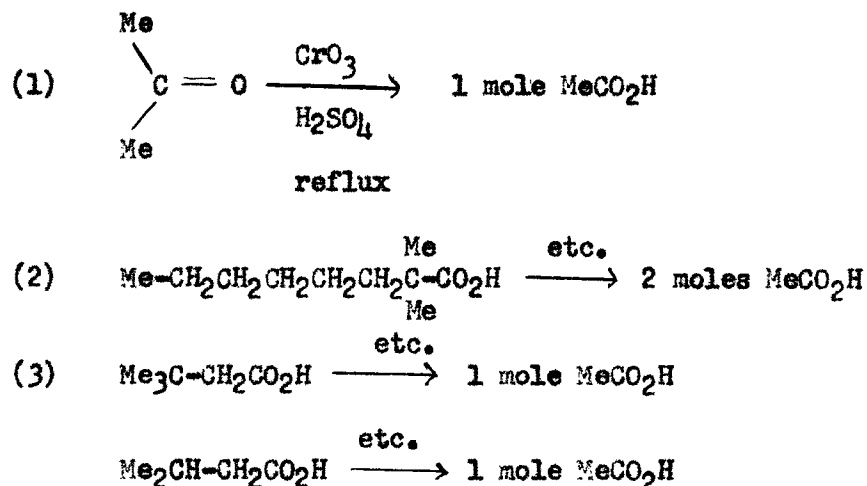
Fig. 8. Reaction Vessel and Spiral Bubbler
Attached in Series to a Mercury Bubbler.

Passage of dry hydrogen chloride through the dried solution resulted in the quantitative precipitation of aniline hydrochloride (yield 50%). This compound was filtered off, dried, and a small sample combusted to CO_2 . The activity was counted as BaCO_3 .

The results obtained are shown on p. 84.

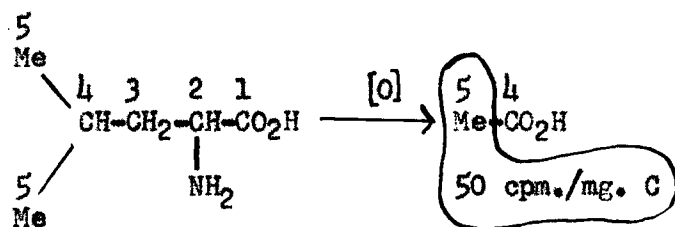
The degradation of leucine

To date there has been no method for the determination of activity in the branched methyls of leucine. It was hoped that the chromate oxidation of Pregl (34), as used by Ginger (22), might prove worthwhile. This method is simply a complete oxidation of the carbon chain with the exception of the two carbon unit, represented by the branched (or end) methyl, and the carbon atom to which it is attached. The method is general but not completely quantitative (31). Some examples of the theoretical yields of acetic acid are shown below.

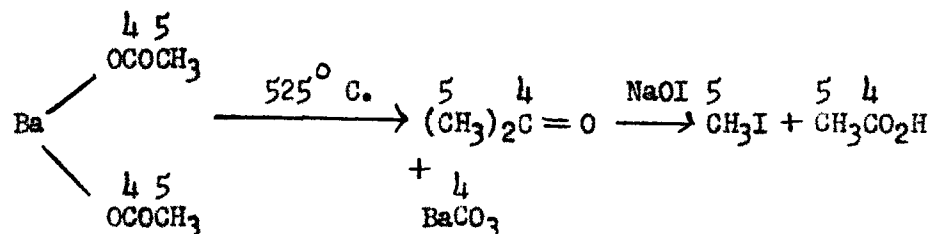


By analogy, one would expect to obtain one mole of acetic acid from one mole of leucine.

There is a limitation in the use of this method, with respect to the isotopic content of the two methyls of leucine. Inasmuch as the oxidation lacks stereospecificity, one cannot expect to obtain data on the C^{14} content of only one of the methyls. For example, if in the course of biosynthesis, only one of the methyls is labeled, with a specific activity of 100 counts per minute/mg. of carbon, one would obtain acetic acid whose methyl carbon would have a specific activity of 50 counts per minute/mg. of carbon.

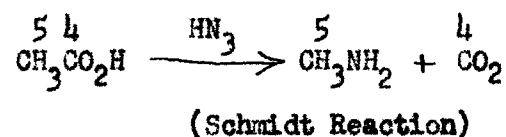
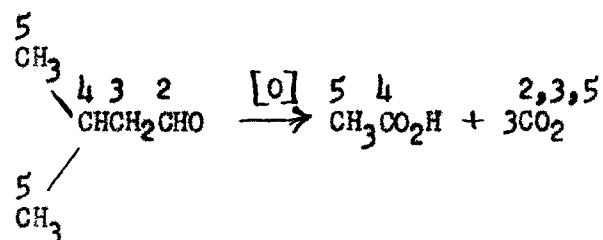
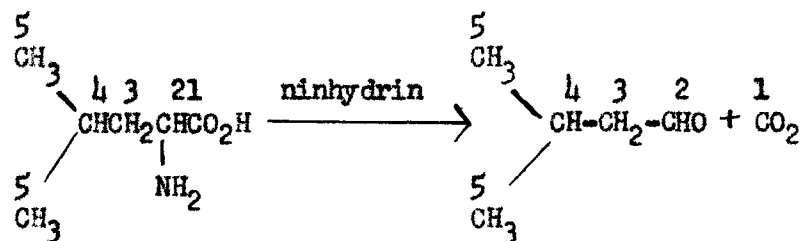


In order to obtain a value for the methyl carbon of acetic acid, further degradation was necessary. This was most simply done by conversion of the acetic acid to the barium salt, followed by pyrolysis to yield acetone. The acetone was further decomposed by NaOI (the Iodoform Reaction) to CHI_3 and acetic acid.



The activity in iodoform represents an average of that in the methyl carbons of the original leucine.

In view of the fact that interest in leucine was motivated by its "uniqueness" of structure, no attempt was made to perform a stepwise degradation of the entire carbon chain. However, such a degradation could have been partially achieved by using the ninhydrin reaction as the first step in the following scheme:



In the present work, only the specific activities of carbons 1, 4, and 5 were determined.

The labeled leucine was eluted from a paper chromatogram with 5 ml. of H₂O. Carrier leucine (200 mg.) was added and dissolved completely by stirring on a steam bath. Absolute ethanol (20 ml.) was added to the hot solution and the mixture was again stirred and heated until solution was complete. After standing in the refrigerator for about one hour, the crystalline leucine was filtered off and dried; yield,

173 mg. (86.5% recovery). Repetition of the crystallization yielded material of constant specific activity. A portion of the recrystallized leucine was converted to CO_2 by means of the Van-Slyke Folch oxidation mixture (11, 46). This activity was counted as BaCO_3 .

A second portion of the recrystallized leucine (50 mg.) was placed in a reaction vessel (Fig. 8) and was dissolved in 2 ml. of concentrated H_2SO_4 .

After cooling this mixture in an ice bath, there was added, dropwise, 5 ml. of 5 N CrO_3 (168 g./l.). The solution immediately darkened as the oxidation began. A stream of CO_2 -free air was slowly pulled through the solution to prevent super-heating and the mixture was gently refluxed with the aid of a water condenser. After approximately 90 minutes, the reaction mixture was cooled and transferred to a steam distillation apparatus (Misco Micro-Kjeldahl Distillation Apparatus). The acetic acid was distilled from the mixture by twice refilling the water bulb with water. It was not found necessary to reduce the excess CrO_3 with hydrazine hydrate prior to distillation. (The overoxidation of acetic acid by CrO_3 should not be serious when using steam distillation.) Titration with

0.01 N $\text{Ba}(\text{OH})_2$ to an end point at pH 7.5^a served to estimate the yield of acetic acid and to convert the acid to the barium salt. The yield of acetic acid from the oxidation was about 75%.

The dilute barium acetate solution was concentrated in vacuo to approximately 0.4 ml. After transferring to a 12 ml. centrifuge tube, absolute ethanol was added until the solution was permanently turbid. Barium acetate was deposited from the solution upon standing. The addition of diethyl ether and heating sometimes facilitated the precipitation.

After centrifugation, the clear supernatant was discarded and the tube was placed in a drying oven. When thoroughly dry, the barium acetate was removed from the tube by means of a small spatula. For the purpose of radio-assay, a weighed portion of the barium acetate was plated (with water) onto a glass plate and counted as such.

The position of radioactivity within the acetate residue was determined by the method of Aronoff, Haas, and Fries (3); pyrolysis

^aIn order to prevent the formation of BaCO_3 during titration and subsequent isolation of the barium acetate, several precautions were taken. An end point with phenolphthalein was not used as this brings the pH to about 10. Instead, a mixed indicator, prepared by mixing equal volumes of 0.1% solutions of the sodium salts of phenol red and brom thymol blue, was used to give an endpoint of pH 7.5. (The color change was yellow below, and purple above pH 7.5.) During titration, the solution was kept hot to lower the solubility of CO_2 , and, during vacuum concentration of the solution, CO_2 -free air was led through the distillation apparatus (Fig. 5). The effectiveness of these precautions in preventing BaCO_3 formation was tested by titrating a known amount of acetic acid-1- C^{14} by the above method. Radioassay of the resultant barium acetate showed that no dilution of activity had occurred.

of the barium acetate to BaCO_3 and acetone. This was followed by treatment of the acetone with NaOI (the Iodoform Reaction) to yield iodoform (p. 48).

Thirty mg. of barium acetate was placed in a $2\frac{1}{2}/40$ standard taper test tube. An outer $2\frac{1}{2}/40$ joint having a sealed in delivery tube, and exit tube, was placed in position. The bottom of the delivery tube was two cm. from the barium acetate. A spiral bubbler containing 10 ml. of water was connected to the exit tube and nitrogen gas was flushed through the system via the delivery tube. After the air had been displaced with nitrogen, the barium acetate was heated at 525°C. for 10 minutes. This temperature was maintained by a small electric furnace controlled by means of a Variac. The tube was allowed to cool for 10 minutes during which time nitrogen was continuously flushed through the test tube and bubbler.

To the dilute acetone solution contained in the bubbler, were added 2 ml. of 5% NaOH followed by dropwise addition of 0.1 N KI_3 until the solution became permanently yellow. Iodoform precipitation occurred almost immediately. This compound was assayed directly by plating a pyridine solution of the compound onto a glass plate. The iodoform activity represented carbon 5 of the leucine chain.

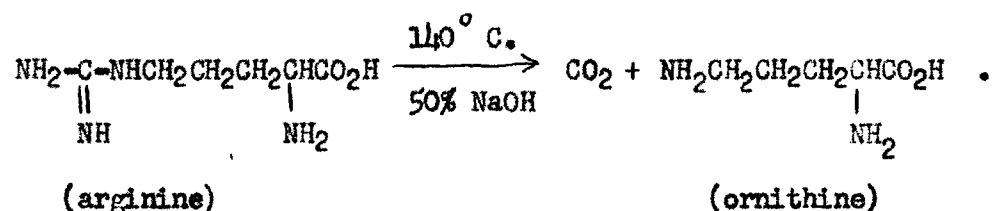
The gray, sintered BaCO_3 was removed from the test tube and purified by regeneration with acid and reprecipitation. This activity represented carbon 4 of the leucine chain.

The specific activity of carbon 1 was measured by the usual ninhydrin method (47) (p. 49).

The above procedure gave the results shown on p.106.

The degradation of arginine

The guanido carbon of arginine was assayed by the method of Strassman and Weinhouse (44);



The paper chromatographically isolated arginine was eluted in a minimum amount of water. Two hundred mg. of inert arginine hydrochloride was dissolved in the eluate. The addition of absolute ethanol served to precipitate the arginine hydrochloride. A single recrystallization yielded material of radiochemical purity. Three mg. of this compound were converted to CO_2 and counted as BaCO_3 .

One hundred mg. of the pure, crystalline arginine hydrochloride was added to 5 ml. of water and 3 ml. of CO_2 -free 50% NaOH in a reaction vessel (Fig. 8). A small water reflux condenser, connected to an Ascarite tower, was fixed in place. The reaction mixture was heated on an oil bath, for 6 to 7 hours, at 140° C. At the end of this period, a spiral bubbler containing 0.1 N NaOH was added to the condenser air outlet in place of the Ascarite tower. Five ml. of 10 N H_2SO_4

was cautiously tipped into the reaction mixture from the side arm. CO₂-free air was drawn through the flask and bubbler while the flask was momentarily brought to a boil. Twenty minutes were allowed for complete collection of the evolved CO₂. After conversion to BaCO₃ the activity was measured and represented the guanido carbon of arginine. The molecule was not further degraded, due to the low activity remaining in the ornithine. The results are reported on p. 101.

RESULTS AND DISCUSSION

Protein Synthesis in Excised and Attached Leaves

One of the most important findings concerning leaf protein metabolism is the observation that leaves, which have been detached from the parent plant, lose protein N to form asparagine and/or glutamine (14). Either of two explanations would serve to elucidate this fact; (1) the rate of protein synthesis has been reduced, or (2) the rate of protein degradation has been increased. A solution to this problem was obviously impossible at the time of the original researches.

With the aid of $C^{14}O_2$, it was proposed to test the two hypotheses. This was done by simply feeding $C^{14}O_2$ to an excised leaf and an attached leaf for the same time interval. At the end of the experiment, both leaves were killed and analyzed for radioactive free amino acids and protein. The finding of labeled protein in the excised leaf would mean that protein had been formed de novo from photosynthate. It was furthermore the purpose to compare the total amount of radioactivity in this protein with that of the attached leaf, and finally, to compare the incorporation of particular radioactive amino acids in the two systems. In the following experiments and discussion, the period of time between excision of the leaf and C^{14} -administration has been abbreviated as E_t (excision time).

Excised soybean leaves

Soybeans (var. Hawkeye) were grown from seed, four seeds being placed in each 6 inch pot. During the entire period of growth, the plants were kept in a special light chamber containing twenty, 20 W. fluorescent bulbs of various colors, so balanced as to produce a range of wavelengths which simulated the solar spectrum. A photoperiod of 14 hours light and 10 hours of darkness was maintained. The leaves and plants selected for all experiments were carefully chosen for vigour and lack of disease. Unless otherwise noted, the plant material was three to five weeks old (from seed) when used.

A series of experiments was run in the following manner:

Two mature (i.e., fully expanded) trifoliolate leaves were excised, one twelve hours and the other six hours before feeding. Both leaves were placed in water and kept under the same conditions.

The leaves were fed $C^{14}O_2$ (3-4 mg. $BaC^{14}O_3$, 7.2 millicuries/millimole) for 1 hour in the light and killed in boiling 80% ethanol.

An attached mature trifoliolate leaf was fed $C^{14}O_2$ under equivalent conditions of light and temperature. Gross analyses of all three leaves are shown in Table 1.

The effect of time of excision is such that relatively more amino acid activity is found in the 80% ethanol-soluble fraction in longer time periods. The significance of this fact is not clear. If it is assumed that the protein turnover time is considerably greater than the duration of feeding (one hour), most of the activity in the 80% ethanol-soluble amino acids cannot be present as a result of

Table 1

Protein Synthesis in Excised and Attached Leaves
After a 60 Minute Photosynthesis in $C^{14}O_2$.

	Attached	$E_t = 6$ hours	$E_t = 12$ hours
Per cent of total amino acid activity in the form of protein	51.2%	40.0%	20.8%

protein hydrolysis. Such amino acids would be, so to speak, "in limbo", as a result of non-incorporation into protein. Therefore, a relative increase in the amount of activity in free amino acids, with increasing time between excision of exposure to $C^{14}O_2$, is best interpreted as loss of capacity to synthesize protein.

The question now arises as to whether or not the non-incorporation of amino acids is due to a selective^a process. In other words, are

^aThe significance of a selective incorporation (or non-incorporation) might lie in the mechanism of protein turnover. If a certain peptide moiety of the protein turned over more rapidly than the rest of the molecule, one might expect the amino acids of the peptide to be replaced by certain amino acids at a rate in excess of the overall incorporation of amino acids. Other explanations, also based on a specific type of turnover, could also apply..

some amino acids allowed to remain in the free state while others are incorporated into protein? This question was partially answered by measurement of the distribution of radioactive amino acids in all three leaves. The data are shown on Table 2.

With the possible exceptions of glutamic acid and glycine, no obvious trend (in the direction of selectivity), is apparent.

In the case of glutamic acid, the results are not conclusive. It has been a common experience in this laboratory to find that very similar appearing leaves may yield somewhat different ratios of radioactive compounds. Properly speaking, this is a matter requiring statistical approach. In view of the apparent variability of leaves, samples containing many leaves would have to be used. Another, more practical, method consists of taking large samples of small leaf punches which have been exposed to $C^{14}O_2$ for a suitable period (p. 72). Evidence for selectivity of turnover rate may, however, be adduced by an experiment of the following type:

Four excised leaves were fed $C^{14}O_2$ for one hour, at which time the chamber was opened to the air. At the end of one hour, four hours, eight hours, and twenty hours, a leaf was removed, killed, and analysed for radioactive amino acids, etc.

An identical experiment was performed with attached leaves.

The most significant data obtained were the percentages of total activity in glutamic acid in the form of protein glutamate. In the

Table 2

Distribution of Amino Acid Radioactivity in Cation
Fractions of Excised and Attached Leaves.

Amino acid ^a	Per cent of total cation activity					
	Attached		$E_t = 6$ hours		$E_t = 12$ hours	
	Cation fraction:		Cation fraction:		Cation fraction:	
	free	protein	free	protein	free	protein
aspartic acid	16.0	9.7	17.0	13.3	18.4	10.2
asparagine	10.2	---	10.4	---	2.38	---
aspartic acid plus asparagine	26.2	---	27.4	---	20.8	---
glutamic acid	13.1	3.3	20.2	4.4	9.4	7.5
glutamine	1.0	---	0.0	---	1.0	---
glutamic acid plus glutamine	14.1	---	20.2	---	10.4	---
glycine	3.2	19.2	12.7	24.0	4.35	32.4
serine	40.8	21.6	31.8	19.5	58.4	18.0
alanine	7.4	12.4	5.4	12.0	4.9	12.0

^aAmino acids other than those shown have been found. Phenylalanine, tyrosine, proline, leucine and arginine have been detected. In all such cases, the combined activities of these amino acids were less than 5% of the cation activity.

case of attached leaves this was a fairly constant quantity, approximately 18%. The results with excised leaves show that the incorporation of glutamate is a function of time.

Table 3 also shows the general increase of protein radioactivity at the expense of the free amino acids. It is seen that the protein glutamate increased at a rate greater than that of the total amino acids into protein.

The cause of such non-uniformity of amino acid incorporation in excised leaves may be one of the following:

(1.) Selectivity in protein turnover rates by means of stimulation or inhibition

- (a) A particular glutamate peptide is rapidly turning over as compared to the rest of the protein, previously mentioned.
- (b) A particular glutamate-rich protein is rapidly turning over as compared to other proteins.

(2.) Net protein synthesis; i.e.:

The increased glutamate uptake may be due to the net synthesis of a glutamate-rich protein.

(3.) Utilization of free glutamic acid in other processes; i.e.:

One might expect that the removal of free glutamic acid (or glutamine) in other ways (i.e., respiration and synthesis

Table 3

The Incorporation of Glutamic Acid into
the Protein of Excised Leaves.

	Per cent of the total cation activity (free plus protein)			
	1 hour PS ^a	4 hour PS	8 hour PS	20 hour PS
Protein glutamate	5.4	57.0	69.0	88.0
Protein	23.0	52.0	59.0	66.4

^aphotosynthesis

of other compounds), would lower the activity of glutamate in the alcohol soluble fraction. This would lead to the reported result which is on a percentage basis (see Table 3). Actually, this is not the case. The fractional and specific activity in free glutamic acid increases with time, p. 75 and 77.

A similar argument applies to the non-utilization of the other free amino acids. In this case, the activity of the free amino acids would increase, thus lowering the percentage activity with which they appear in protein. It was found, however, that the percentage of activity in the ethanol-soluble cation fraction (on the basis of total ethanol-soluble activity) was relatively constant in all experiments. The percentage of protein activity, on the basis of total ethanol insoluble activity, is likewise a fairly constant figure (approximately 15%) for mature leaves. This would mean that the transformation of free amino acids to materials other than protein was independent of excision. One may thus conclude that the discrimination against specific free amino acids in protein synthesis is not a result of processes other than protein synthesis or turnover.

In order to localize the excess activity in protein glutamate, and to localize the activity of the other amino acids, further data would be needed. For this purpose, the most useful method would be a determination of amino acid turnover times in isolated leaf proteins.

The next section describes an attempt in this direction. The radioactive leaf was ground and the protein was extracted. Paper electrophoresis was used to isolate the individual proteins.

An attempt to determine turnover times of individual leaf proteins

For the determination of turnover time of any metabolite, it is imperative to be able to separate the given radioactive species from all other radioactive materials. In the case of plant proteins there are operational difficulties, not the least of which is the lack of a simple method for the isolation of a single protein species. Past methods have not only proven uncertain and of low yield, but are also inapplicable on a micro-scale. The problem is simply that of being able to isolate a given protein from a single radioactive leaf or leaf punch.

From the previous discussion (p. 4), it seemed reasonable to assume that the most fitting subject for isolation was the "Fraction I" (41) component of cytoplasmic protein. By means of paper electrophoresis, it was hoped that a homogeneous radioactive product could be obtained. The method used is given below.

At the end of a feeding experiment, the leaf was immersed in N_2 (liquid) and ground to a fine powder with the aid of a stirring rod. The powder was then extracted (at $4^\circ C.$) with 0.25 M sucrose plus 0.05 M phosphate buffer (pH 6.8). In more recent experiments, the leaf was simply ground in 2 ml. of the sucrose-phosphate medium (at $4^\circ C.$) without prior freezing.

Medium speed centrifugation brought down the cell debris leaving a green suspension.

This supernate contained chloroplast fragments, starch, cytoplasmic proteins and, of course, the water soluble sugars, amino acids, and organic acids.

Following dialysis^a against water at 1° C., the suspension was lyophilized, resuspended in about 50 microliters of water, and placed on a band at the origin of the filter paper strip (six inches wide).

The apparatus (Shandon Ltd., London, England) is a simple Plexiglass box containing carbon electrodes and compartments for the buffer. The paper strip is suspended from a thin wire and dips into each compartment, thus making the electrical contact. A rectifier unit provides a maximum voltage of 150 V. (D. C.) from the normal 120 V., A. C. line.

Inasmuch as the rate of protein movement is somewhat dependent on the amount of protein extract placed at the origin, it was found convenient to use a marker whose movement would act as an indicator of migrational velocity. For this purpose, the dye, "Fast Green", was employed.

^aDialysis is not normally required prior to paper electrophoresis. This is a convenient feature of the apparatus. However, in the present case, it was known that a number of very radioactive anionic materials would migrate to the same position as the leading protein component.

An initial voltage of 120 V. was set on the electrophoresis chamber and the current was allowed to flow for a suitable time (8-12 hours).

The particular buffer used will depend on the protein one wishes to isolate. In the case of blood plasma proteins, excellent resolution is obtained with 0.1 M barbiturate buffer (pH 8.6) (20). The leaf proteins streamed badly in this buffer and were apparently better separated with 0.1 M maleate buffer (pH 7.0) although the rate of migration was slower.

The paper was removed and air-dried at room temperature. The location of the protein bands was determined by cutting off a thin section of the paper and treating it with a protein stain: bromphenol blue in 95% ethanol saturated with mercuric chloride (8). After washing in tap water, the proteins are visible as blue bands on a white background.

The results of this method on leaf proteins are, at present, difficult to assess.

Due to the low concentrations of protein used, and probably due to some structural or compositional feature, the leaf protein having the greatest mobility was only lightly stained with the staining reagent. This led to some uncertainty with regard to its position on the paper, and to whether or not it was a single band or a number of bands which had streamed together.

On the basis of migrational velocity and the means of preparation, it was felt that the fastest and most discrete protein band obtained was probably "Fraction I" protein. Before this could be definitely stated, one would have to make a co-electrophoretograph of the radioactive material plus some genuine "Fraction I" protein as prepared by ultra-centrifugation (41).

In the present investigation, the band, tentatively named "Fraction I" protein, was cut out and hydrolysed in the usual manner. Unfortunately, the buffer still remaining in the paper rendered the hydrolysate unfit for paper chromatographic isolation of the resulting amino acids. In future work it will be necessary to dialyse first the paper strip in order to remove extraneous materials.

Due to the low level of activity and the uncertainty of isolation and identification, the present work was not successful. However, it is felt that this approach is correct. Future work on the separation of leaf proteins via paper micro-electrophoresis seems definitely worthwhile.

Another approach to studies on leaf protein synthesis and turnover appears to be feasible at this time, viz.; the synthesis and turnover of Tobacco Mosaic Virus (TMV). This material recommends itself for two reasons.

(1.) Methods for the administration of tracers other than

$C^{14}O_2$ have been worked out by Commoner, et al. (16).

In addition, sampling may be made statistically valid by floating many leaf punches on the labeled substrate.

- (2.) A method for the isolation of TMV via paper chromatography has been perfected by Cochran (15).

Insofar as TMV is an abnormal leaf protein, it may be argued that little concerning normal leaf protein may be thus learned. At the present time, this argument is unanswerable.

Excised tobacco leaves

During "short time excision" (i.e., $E_t \approx 6$ to 12 hours; see p. 56), amino acid incorporation occurred at rates somewhat less than that observed in attached leaves. It was felt desirable to determine the rate of amino acid incorporation a number of days after excision. The results of Chibnall (14) and others (49) show the relative amounts of free and protein nitrogen to be affected greatly only after several days. One may therefore ask if amino acid incorporation ceases entirely after "long term excision". Inasmuch as soybean leaves had never been studied in this connection, it was decided to use tobacco leaves. Another factor which influenced this decision was the rapid formation of roots on excised soybean leaves. Two to three weeks following excision these leaves developed a visible root system. If the effects of excision are due to the absence of root tissue, one obviously cannot perform reliable experiments with soybean leaves after a few days excision. Even in the absence of visible roots, one

cannot be sure that primordial root tissue is absent. Although tobacco may also form roots, the validity of excision phenomena with these leaves has been established (49).

A single mature tobacco leaf was carefully excised and placed in nutrient solution (Hoagland's Solution No. 1) (25). The leaf was kept under normal illumination and the nutrient solution was changed every two days. After eight days, the cut petiole was trimmed to expose a fresh portion of stem tissue. Simultaneously, an attached leaf was excised and placed in tap water with the eight-day old leaf. Both leaves were allowed to equilibrate. The leaves were then exposed to $C^{14}O_2$ for one hour in the light. After killing, the leaves were analysed in the usual manner (Fig. 9, 10, and 11).

The total uptake of $C^{14}O_2$ was roughly equivalent in both leaves. The percentage of protein activity (based on the total activity in amino acids) was 54.8% for the attached leaf and 39.0% for the excised leaf.

Protein synthesis occurred eight days following excision ($E_t = 8$ days) at a somewhat slower rate than that in a freshly excised ($E_t = 0$ days) leaf. Although this is the trend observed in "short time excision", it is rather surprising that the loss of amino acid incorporation power is not greater during the eight day period. It is possible that the rate of amino acid incorporation quickly reaches

alanine

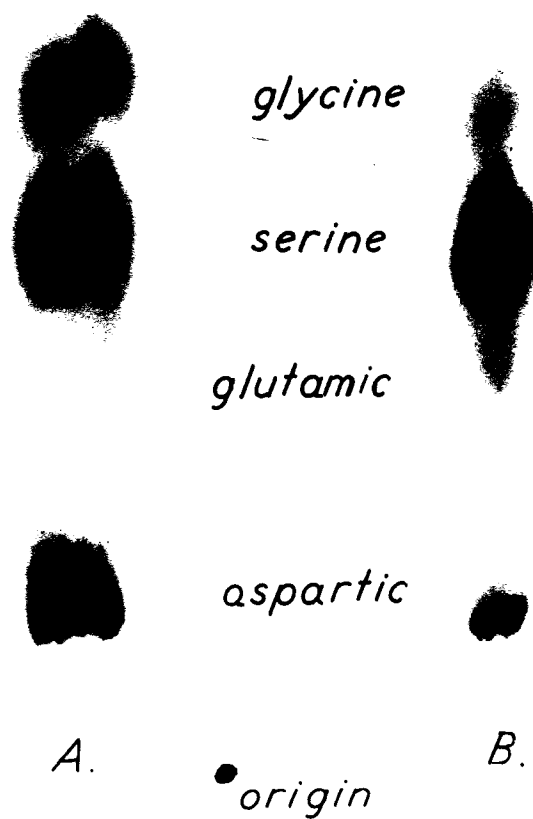


Fig. 9. Radioautographs of Free Amino Acids Produced
During 1 Hour Photosynthesis by Tobacco Leaves:
A. $E_t = 8$ days, B. $E_t = 0$ days.

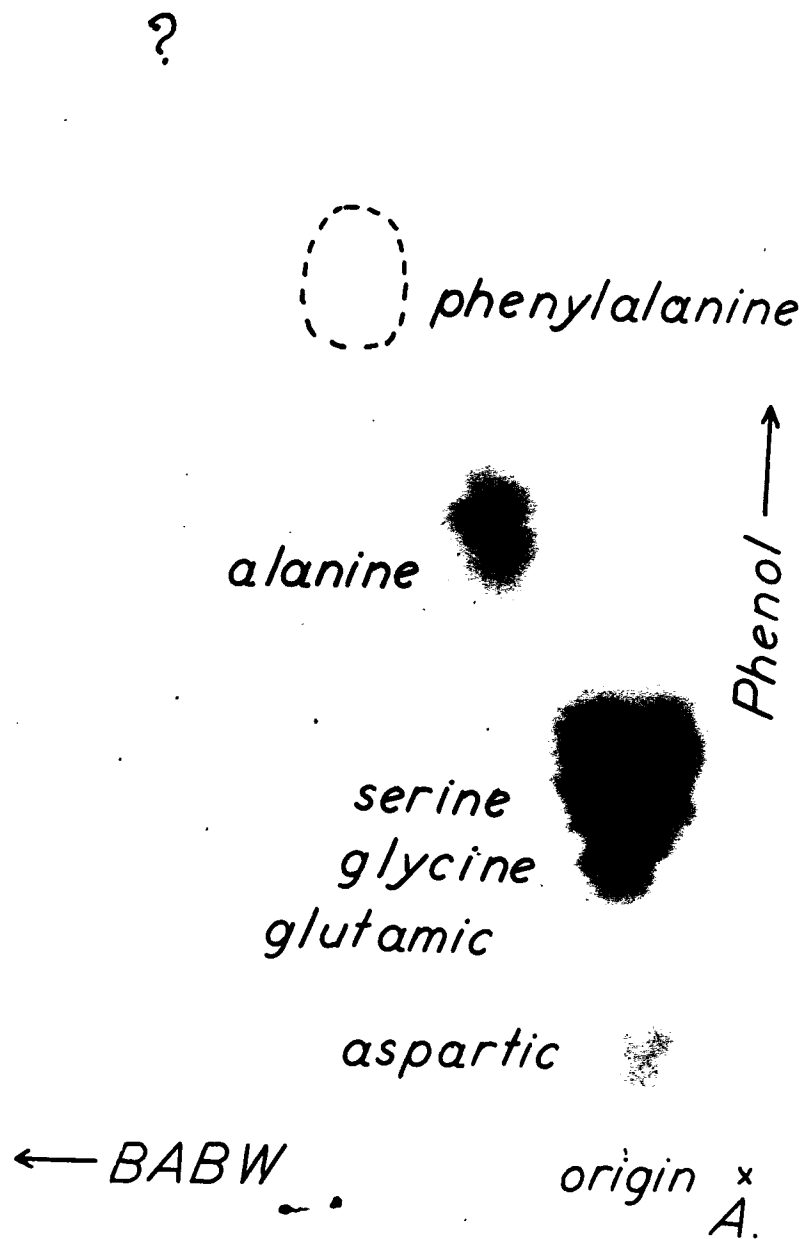


Fig. 10. Radioautograph of Protein Amino Acids Produced During
One Hour Photosynthesis by a Tobacco Leaf, $E_t = 8$ days.

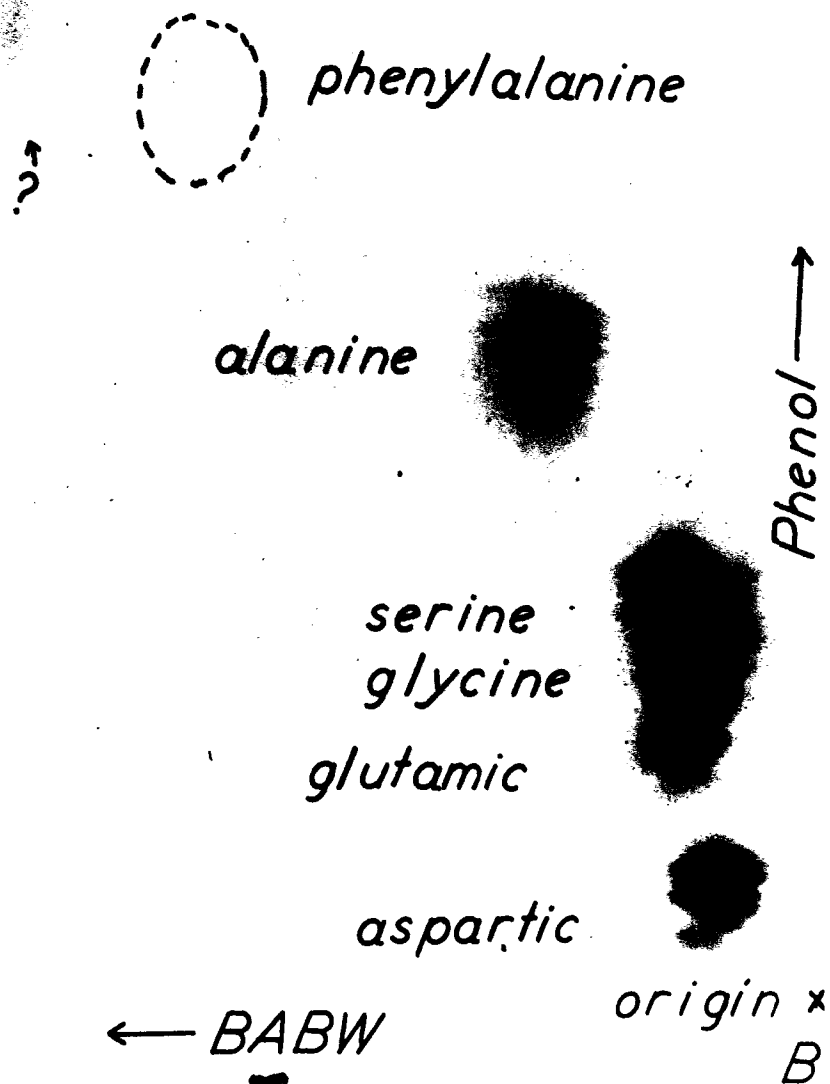


Fig. 11. Radioautograph of Protein Amino Acids Produced During
One Hour Photosynthesis by a Tobacco Leaf, $E_t = 0$ days.

a lower but fairly stable limit following excision. It is also possible that a quantitative comparison of tobacco and soybean leaves is not valid.

A comparative tabulation of the distribution of amino acid radioactivity is shown in Table 4. It will be noted that there has been no preferential incorporation of glutamate. Both leaves have essentially the same ratio between the free and protein glutamate activity. The only apparent difference is in the incorporation of aspartate. This compound had a free aspartate to protein aspartate ratio of 1.33 in the attached leaf and 3.0 in the excised leaf. However, in view of the differences in supplied mineral nutrients, definite conclusions cannot be made.

Amino Acid Turnover in Leaf Punches

The aforementioned variability of individual leaves (p. 58) and the desirability of working with small, rather than large, amounts of radioactivity forced a search for other methods of leaf sampling. Several experiments were attempted with large numbers of small leaf punches and with tissue free suspensions of chlorenchyma (i.e., the thin-walled, chlorophyll containing cells). Presumably, these are the most active physiological units in leaves.

It was previously shown, though not emphasized, that the activity in glycine and serine accounted for the bulk of the activity converted into protein during a one hour photosynthesis (p. 59). However, these experiments were performed with single leaves and the

Table 4

The Incorporation of $C^{14}O_2$ into Free Amino Acids and
Protein of Excised and Attached Tobacco Leaves
During One Hour Photosynthesis.

Amino acid	Per cent activity of the cation fractions			
	Attached ($E_t = 0$ days)		Excised ($E_t = 8$ days)	
	Cation fraction:		Cation fraction:	
	free	protein	free	protein
aspartic acid ^a	18.0	13.6	30.0	10.0
glutamic acid ^a	11.0	6.5	7.5	4.7
glycine	9.1	27.0	17.0	33.0
serine	43.0	22.1	34.0	21.0
alanine	7.8	15.4	6.6	17.0
phenylalanine	4.1	ca. 2.0	2.5	trace
tyrosine	none	ca. 2.0	trace	trace
unidentified	trace	10.0 ^b	trace	11.2 ^b

^aThe free amino acids were hydrolysed prior to chromatography. The activity, normally found in asparagine and glutamine, appeared in the free acids.

^bThis material did not correspond to any known position on the papergrams. It is apparently homogeneous and may be a purine or pyrimidine released by hydrolysis of nucleoprotein. Its location is shown on fig. 10 and 11.

data could not show which of these two amino acids were incorporated at the faster rate. The following experiments provided larger samples and were therefore meaningful. Another improvement was made. Short term exposure to $C^{14}O_2$ was used. This meant that the leaf tissue was exposed to the atmosphere for a relatively long portion of its experimental life and thus normal behavior could be observed.

Leaf punches were carefully cut with the aid of a sharp 9 mm. cork borer. The punches were placed in a closed petri dish whose bottom was lined with wet filter paper. The punches were exposed to subdued light for twelve hours in order to attain a relative steady state.

Forty of these leaf punches were symmetrically arranged, in the illuminated plastic feeding chamber, upon pieces of damp filter paper. The punches were then exposed to $C^{14}O_2$ for five minutes. At the end of this time, the system was opened and the excess $C^{14}O_2$ removed by short, repeated applications of a slight vacuum. Fifteen minutes after the experiment began, twenty punches were removed, killed, and assayed. The remaining punches were similarly treated after fifteen more minutes had elapsed. The pertinent data is shown in Table 5.

Examination of this table shows that glycine is first incorporated at the highest level. Serine is rapidly incorporated so that its activity is equal to that of glycine in 30 minutes. The total amount of glycine activity was 53,000 counts per minute in the 15 minute period, and 89,000 counts per minute in the 30 minute period. The

Table 5

Distribution of Radioactivity in Free and Protein
Amino Acids after a 15 and 30 Minute
Photosynthesis with Leaf Punches.

Amino acid	Per cent activity of the cation fractions			
	15 minute photosynthesis		30 minute photosynthesis	
	Cation fraction:		Cation fraction:	
	free	protein	free	protein
aspartic acid	18.7	9.6	15.2	7.9
asparagine	0.0	---	3.2	---
glutamic acid	3.4	0.0	11.7	0.0
glutamine	0.0	---	0.0	---
serine	44.0	20.0	38.0	26.0
glycine	5.0	38.5	12.6	26.0
alanine	26.8	22.0	15.2	19.6
phenylalanine	1.6	0.0	1.8	3.3
tyrosine	0.0	0.0	1.0	2.8
leucine	0.0	0.0	0.0	2.3
others ^a	0.0	10.0	0.0	9.8

^athese materials were not identified, the activity being distributed among four or five compounds

corresponding level of serine was 125,400 counts per minute in the 15 minute and 147,000 counts per minute in the 30 minute period. One unusual aspect of these data is the non-incorporation of glutamate into protein. It will be remembered that in the reported work on excised soybean leaves, glutamate was incorporated at a level above that of the other amino acids (p. 61).

The fact that this does not occur here seems evidence for a difference in the metabolism of excised leaves and leaf punches. However, the comparison is unfair in that the experiments were performed for different time periods.

In order to obtain evidence on the rates of turnover of the free amino acids another experiment was performed. In this case specific activities were determined. This, of course, allows one to take account of pool size and is a more reliable index of rate processes.

Leaf punches, obtained and treated in the usual manner, were exposed to $C^{14}O_2$ for two minutes. The system was cleared of excess $C^{14}O_2$ as described above. A sample consisting of fifteen punches was removed, killed, and assayed at the end of five, fifteen, thirty, and sixty minutes. The concentration and radioactivity of each amino acid was determined from paper chromatograms. The results are shown in Table 6.

Several of the observed relationships are worthy of comment. In five minutes, the free amino acid of highest specific activity was alanine. This was also the amino acid having the highest observable

Table 6

The Specific Activities of Amino Acids at Timed Intervals
Following Exposure to $C^{14}O_2$ in the Light.

Amino acid	Specific activity ^a			
	5 minute	15 minute	30 minute	60 minute
	PS ^b	PS	PS	PS
Aspartic acid	72.0	85.4	75.6	69.2
Asparagine	0.0	22.6	23.6	71.5
Glutamic acid	9.2	71.0	100	152
Glycine	37.4	31.2	22.4	31.2
Serine	336	219	120	124
Alanine	700	214	59.5	38.2

^ain counts/minute/microgram of carbon

^bphotosynthesis

turnover rate. Serine followed, and was closely related to the alanine turnover. It is likely that they share a common precursor which is directly associated with photosynthesis and is rapidly turning over. In view of the already established relationship between alanine and glyceric acid (35), this is not a surprising result. The turnover of alanine is most probably associated with that of glyceric acid. It is likely that the two compounds are in equilibrium, so that the activity originally transferred to alanine is shifted back to glycerate as well as into protein. A similar argument applies to serine.

It is also interesting to note the apparent precursor-product relationship existing between aspartic acid and asparagine.

The situation with reference to glycine is probably more complex. Although we have seen that this compound is rapidly converted to protein, no indication of its rapid turnover is shown in Table 6. It is likely that glycine is being rapidly utilized, not only in protein synthesis, but also in the formation of serine (30), and that the glycine is therefore being synthesized from a large pool of slowly turning over precursor. It is likely, too, that this precursor is not in a steady state, being synthesized more rapidly than its rate of breakdown. The largest scale donor of glycine activity is probably glucose which is itself in equilibrium with an enormous starch pool.

The close relationship of aspartate, glutamate, and glycine to glucose, and the fact that the position of radioactivity within the

molecules were not taken account of, leaves one in doubt as to the actual paths taken by these compounds. For example, one might consider the simplest case; that of glycine. The work of Vernon and Aronoff (47) has shown that the two carbons of this compound are equally labeled in short time photosynthesis. If glycine is converted to serine during short time photosynthesis, the resultant serine will have a lower specific activity than that serine formed directly from glyceric acid. In long time experiments (in which $C^{12}O_2$ has finally replaced $C^{14}O_2$ as the substrate), the serine derived from glycine would be "hotter" than that derived from glyceric acid. This would arise by virtue of glycine being formed from the large pool of radioactive glucose. The overall effect would be a flattening-out of the serine specific activity curve. What then may be said concerning the relative intimacy of serine and alanine with the primary photosynthate? Serine may be the more closely related to glycerate even though its specific activity curve does not suggest this. Obviously, one can say very little without a full knowledge of all possible pathways and their relative rates. The amassing of such knowledge can only be partially gained by $C^{14}O_2$ experiments. The use of a more specific substrate is definitely called for.

With this in mind, it was decided to attempt the feeding of glycine-2- C^{14} . In this preliminary experiment, it was hoped to determine whether or not glycine is a truly dynamic metabolite in leaves.

The Fate of Leaf Glycine

For this experiment a single, excised tobacco leaf was chosen. (It was apparent from earlier work that tobacco leaves took up water more rapidly than soybean leaves. A more efficient feeding could thus be performed with this subject.) The illuminated leaf was fed with 1-2 mg. of glycine-2-C¹⁴, one hour following excision. The experiment was terminated after four hours. The following gross analysis shows that the glycine was rapidly utilized:

activity in the 80% ethanol extract = 248,000 counts per minute

activity in the hydrolysate of the ethanol-insoluble residue

= 400,000 counts per minute

activity in the crude fiber (17.5 mg.) = 19,840 counts per minute

An analysis of the hydrolysate showed the following distribution:

activity in starch = 280,000 counts per minute,

activity in protein = 120,000 counts per minute.

The distribution of activity in the above protein is shown in Table 7.

It is evident that glycine is metabolized at a rate in excess of its direct conversion to protein. The major utilization of its activity is in the formation of starch. This is a result reminiscent of that found in animals (5). The most likely explanation for this behavior is that the glucose to glycine conversion is an equilibrium process (i.e., reversible).

Table 7

The Distribution of Amino Acid Radioactivity in
Tobacco Leaf Protein Following the Uptake of
Glycine-2-C¹⁴ in the Light.

Amino acid	Per cent activity in the protein
aspartic acid	9.4
glutamic acid	10.3
serine	26.2
glycine	22.5
alanine	11.7
phenylalanine	4.7
leucine	5.6
others ^a	9.4

^aroughly equal distribution between tyrosine, valine, proline, arginine, and cystine (not positively identified)

The glycine to serine conversion has occurred to the extent that the protein serine has a higher total activity than the protein glycine.

The methylene carbon of glycine is an effective precursor of many of the identified amino acids. An interesting fact in this connection is the relatively large amount of glycine activity incorporated into leucine. (Work with $C^{14}O_2$ exposed leaves revealed little activity in leucine.) Other workers (18) have demonstrated the effectiveness of acetate as a leucine precursor in a yeast, Torulopsis utilis.

In order to determine whether or not the incorporation of the methylene carbon is preceded by decomposition of the glycine to two, one-carbon units, the hydrochloride of methylamine- C^{14} was administered. (This compound could arise directly by decarboxylation of glycine.) However, the feeding of this substrate did not result in the labeling of any of the common metabolites. The activity was present in only one compound; possibly an alkaloid. Further work was not performed.

The Synthesis of Phenylalanine in Leaves

It is a well recognized fact that certain amino acids possess unique structures which cannot be readily formed from carbohydrate in the animal body. In two cases, tyrosine and phenylalanine, the "uniqueness" is represented by an aromatic ring.

Green plants obviously possess the ability to form these, as well as all other, amino acids. It is therefore necessary for plants to have an enzyme system (or systems) capable of transforming the photosynthate (carbohydrate) to aromatic structures. Of interest in this respect is the site of such transformations. One should like to know whether the total synthesis can occur in leaf tissue or whether a necessary precursor is formed by the root system. For this purpose, one hour $C^{14}O_2$ exposures were made with illuminated, excised leaves. The leaves used were a soybean leaf excised twelve hours before, and a tobacco leaf excised eight days before the experiment. The isolation and degradation of phenylalanine was performed as described on p. 43. The results are shown in Table 8 and indicate that the ring may be totally synthesized in the leaf (from CO_2). A discussion concerning the mechanism of phenylalanine synthesis is not warranted due to its approximately uniform labeling. It is, however, interesting to speculate on its origin from a seven carbon compound such as the metabolically significant sedoheptulose via shikimic acid.

A Tissue Free Homogeneous Cell Preparation

In order to expedite the feeding of labeled substrate, and to insure statistical sampling, a new method was evolved.

It was found in this laboratory that soybeans, suitably ground in buffer, yielded a very large number of apparently intact parenchyma cells. (These cells seem to be but loosely "stuck together"

Table 8

The Distribution of Radioactivity in Phenylalanine
 Synthesized by Excised Leaves During
 One Hour in $C^{14}O_2$.

Position of activity ^a	Specific activity (in counts per minute per mg. of carbon)	
	Soybean leaf	Tobacco leaf
	($E_t = 12$ hours)	($E_t = 8$ days)
phenylalanine (total)	5.8 ± 0.3	6.0 ± 0.3
carbon 3 plus ring (benzoic acid)	5.3 ± 0.2	5.7 ± 0.3
ring (aniline)	5.6 ± 0.6	5.5 ± 0.6
carbon 3	3.5 ± 0.6^b	9.0 ± 0.7^c
		7.0 ± 0.6^b

^a numbering used is as follows:
$$\begin{array}{c} 3 \quad 2 \quad 1 \\ C_6H_5CH_2CHCO_2H \\ | \\ NH_2 \end{array}$$

^b determined by difference between benzoic acid and aniline

^c determined by polyphosphoric-hydroxylamine method, p. 45

and are easily squeezed through the ruptured epidermis. The same is not true of tobacco leaves. In this case, an identical grinding results only in broken cells and particulate matter.) Inasmuch as these cells could be suspended in a liquid medium, both feeding and timed sampling of a homogeneous population could be effected very simply. With this in mind, extensive investigations were carried out to test the feasibility of the method.

Soybean leaves were carefully ground in 0.25 M sucrose and 0.05 M phosphate buffer (pH 6.8) at about 4° C. A test tube homogenizer with a loosely fitting pestle was used and the resulting suspension was filtered through bolting silk (roughly 100 threads/inch). The filtrate was centrifuged at very low speed (about 80 revolutions per minute) for several minutes. (It was found that centrifugation at higher speeds resulted in almost complete loss of the ability to photosynthesize.) Upon discarding the supernatant and resuspending the cells in nutrient solution (Hoagland's No. 1 or etc.), a tissue-free preparation of intact parenchyma cells was obtained (see Fig. 12).

An illuminated, fresh cell preparation was capable of fixing C^{14}O_2 ^a at a rate corresponding to one-fifth that of a normal leaf on the basis of chlorophyll concentration. The intact leaf and the cell

^aFeeding was accomplished in a thermostated, shaken Warburg vessel. $\text{NaHC}^{14}\text{O}_3$ was simply tipped into the suspension from the side arm.

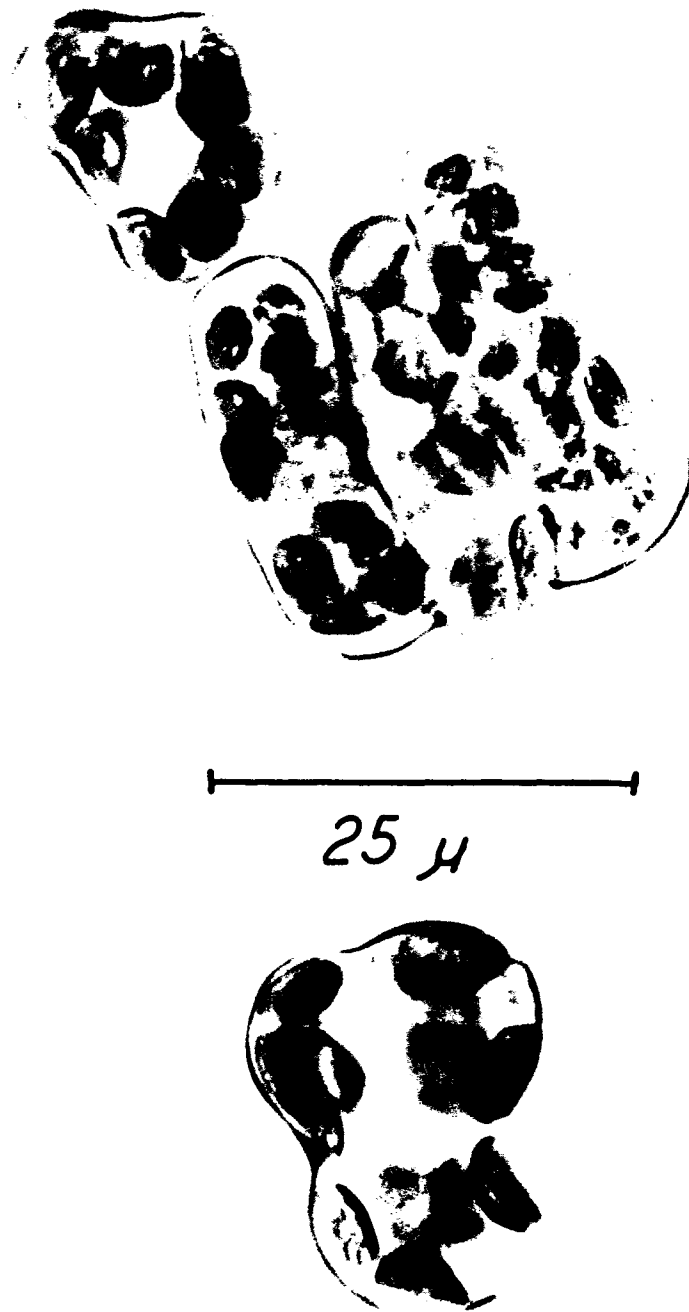


Fig. 12. Photomicrograph of Soybean Leaf Cells.

suspension showed qualitative equivalence in the formation of soluble products (Fig. 13) and starch- C^{14} , but were vastly different in their abilities to synthesize protein.

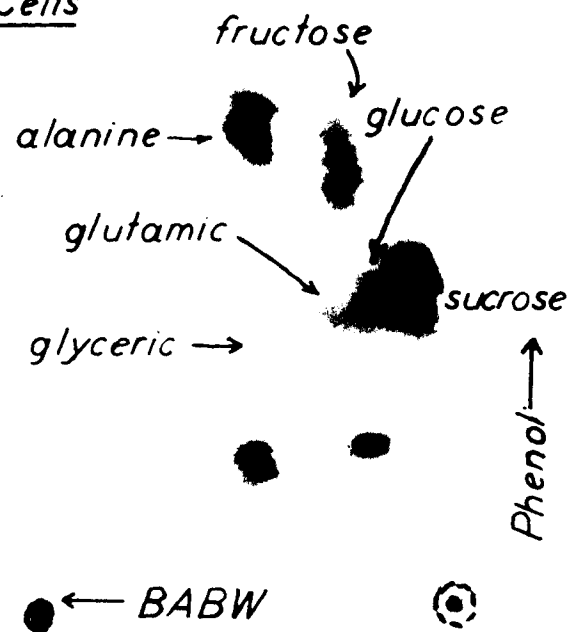
A leaf formed about 15% of the total 80% ethanol-insoluble material as protein while the cell preparation formed essentially none. The latter system was subjected to various time, temperature, and nutrient conditions without finding appreciable radioactivity in the protein. The following is a partial list of attempted variations:

- (1.) Time varied from 1 minute to 8 hours
- (2.) Temperature varied from 21.6° - 26.6° C.
- (3.) Nutrient solutions:
 - (a) Hoagland's No. 1
 - (b) Hoagland's No. 1 plus 0.002 M NH_4
 - (c) Hoagland's No. 1 plus 0.002 M NH_4 plus 0.05 M phosphate buffer (pH 6.8)
 - (d) (c) plus 0.25 M sucrose
 - (e) (c) plus 0.02 M amino acid solution (a mixture of all normally occurring amino acids in the proportions with which they occur in leaf protein).

Washing the cells thoroughly by resuspending four times in fresh solutions also had little effect.

Neither glycine-2- C^{14} nor glucose- C^{14} were used detectably by the cells.

30 min. PS Soybean
Leaf Cells



30 min. PS Soybean
Leaf

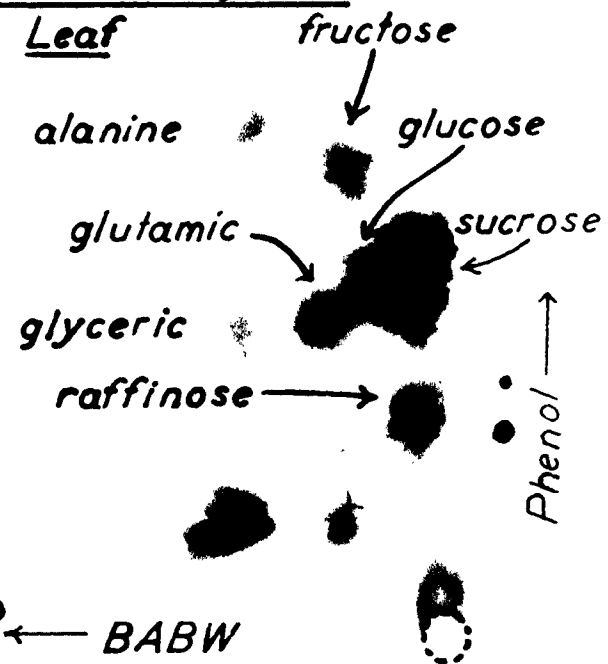


Fig. 13. Radioautographs of Photosynthetic Products
Formed by Leaf Cells and a Leaf.

The above facts could be explained if the cells obtained by grinding were an atypical sample of those in the leaf (for example, if only senescent, low protein forming cells were obtained). That absence of protein synthesis was not an artifact, arising from the method of preparation, was shown as follows.

An intact leaf was exposed to $C^{14}O_2$ in the light for two hours. A cell suspension was then made from one-half of the leaf by the usual technique. The ground residue, the cell suspension (supernatant and cells) and the intact leaf half were analyzed for radioactivity. The results are shown in Table 9^a. It is evident that the cells obtained are capable of protein synthesis in their normal environment. These cells accounted for roughly 86% of the total protein radioactivity in the ground leaf-half.

During a 30 minute photosynthesis in $C^{14}O_2$, a cell suspension had accumulated approximately 35% of its 80% ethanol-soluble radioactivity in the form of the amino acids; alanine, glutamine, aspartic acid, glycine, and serine. It is thus certain that the failure of the cell preparation to form protein is not correlated with the inability to form amino acids from photosynthate. The system is therefore

^aAlthough two halves of the same leaf need not be identical, it nevertheless appears strange that the activity of the 80% ethanol insoluble materials should be so divergent. The much lower total activity in the insoluble materials of the isolated leaf fractions, leads one to suspect that the grinding operation releases large amounts of newly formed starch.

Table 9

The Distribution of Radioactivity in Isolated Leaf
Fractions and Intact Leaf Tissue Following a
Two Hour Exposure to $C^{14}O_2$ in the Light.

Distribution	Radioactivity in counts/minute			
	Cells	Supernatant ^a	Leaf residue	Intact leaf
80% ethanol soluble:	1,200,000	2,000,000	1,000,000	4,700,000
neutral plus anion	1,133,000	1,820,000	955,000	3,410,000
cation (free amino acids)	67,000	70,000	45,000	290,000
80% ethanol insoluble:	892,000	-----	816,000	3,290,000
neutral plus anion	492,000	-----	752,000	2,716,000
cation (protein)	400,000	-----	64,000	574,000
crude fiber:	<u>59,000</u>	<u>-----</u>	<u>250,000</u>	<u>423,000</u>
total:	2,151,000	2,000,000	2,066,000	8,413,000

^aPresumably materials in the clear supernatant would be soluble in 80% ethanol. An exception to this, however, would be the cytoplasmic protein released by rupture of some cells during grinding.

unique in that it demonstrates protein synthesis to be a more sensitive reaction than photosynthesis. Concerning this sensitivity, it is interesting to note that the amino acid incorporating system of Peterson and Greenberg (24) rapidly lost its activity, even at 0° C.

The nature of the malfunction, whereby parenchyma protein synthesis ceases, is of considerable interest. Such knowledge might make possible an understanding of the normal mechanism of amino acid incorporation in leaves. Unfortunately, another physiological malfunction occurs a short time after preparation. It was observed that the rate of photosynthesis (as measured by $C^{14}O_2$ pickup) decreased rapidly with the age of the preparation, without visible sign of cellular deterioration. The loss in the ability to assimilate $C^{14}O_2$ was essentially logarithmic, only 20% of the original rate remained after one hour at 23° C. (in low light intensity). It is, of course, possible that the same factors which negate protein synthesis are also responsible for the degradation of the photosynthetic system.

Despite the failure of this system to yield practical results, it is felt that further work in this direction may be justified. The maintenance of a normal physiological response in the free cells would make possible the sort of approach which has proven so profitable in the animal field.

Amino Acid and Protein Synthesis in the Dark

A vital consideration in protein synthesis by leaves, is the role played by light. Light may stimulate protein synthesis by providing substrate and energy. Whether the energy is supplied by direct conversion of photons to peptide bond energy, or by respiratory energy provided by photosynthetically formed molecules, is a matter of some interest.

There is clear evidence that ATP may supply the energy for the primary formation of peptide bonds (43)^a. If this is assumed to be the case in leaf protein synthesis, one might expect that the absence of light would not diminish the rate of incorporation of amino acids into protein. Goodman, Bradley, and Calvin (23) have shown that ATP is utilized more rapidly in the light than in dark. The phosphorylation of photosynthetic intermediates and formation of polysaccharides evidently makes heavy demands on the ATP pool. It is thus conceivable that light could decrease the rate with which a particular amino acid is incorporated into protein.

A somewhat secondary purpose of this experiment was to determine the capabilities and limitations of dark reactions in the synthesis of amino acids. For example, it was hoped to learn whether all the

^aA possible mechanism is the initial formation of an amino acid phosphate. Black and Gray (6) have recently demonstrated an ATP requiring enzyme system which produces aspartyl phosphate from aspartic acid.

essential amino acids could be thus synthesized. Anfinsen, et al. (2) found that $C^{14}O_2$ incorporation into liver slices resulted in labeled aspartic acid, glutamic acid, and arginine. Other workers (45) have, in addition, found labeled proline, alanine, serine, and glycine. Similar experiments (1) performed with E. coli, demonstrated the occurrence of radioactive aspartic and glutamic acids, arginine, lysine, proline, and threonine. In none of the reported instances have the branched chain and aromatic amino acids been found with appreciable activity.

A trifoliolate soybean leaf was excised six hours prior to feeding. The petiole was kept in Hoagland's No. 1 solution (25) before and during feeding. One hour before feeding commenced, the leaf was inserted into a glass feeding chamber and placed in complete darkness. $C^{14}O_2$ was fed into the system. At the end of 20 hours, the leaf was killed, extracted, and analysed. The gross assay of this material is shown in Table 10. These data show a preponderance of amino acids in both of the main fractions. The ratio of protein activity to free amino acid activity is 0.475. The corresponding photosynthesis experiment (20 hours in the light) reported on p. 58, gave a ratio of 1.97.

It would thus appear that leaves in the light make better use of their free amino acid pools. In order to determine whether or not the

Table 10

Distribution of Radioactivity in a Soybean Leaf
After 20 Hour Dark Fixation with $C^{14}O_2$.

Distribution	Activity (counts/minute)
<hr/>	
80% ethanol soluble:	160,000
cation	122,000
neutral plus anion	38,000
80% ethanol insoluble:	64,000
cation	58,000
neutral plus anion	6,000
crude fiber (5.5 mg.)	2,000

low utilization of amino acids holds for all the amino acids, the appropriate ratios of the two leaves have been compared (Table 11).

From an examination of Table 11, it is apparent that the effect is non-uniform. The leaf which had undergone the dark fixation had lowered ratios for aspartic, glutamic, serine, and probably alanine. However, in this leaf, the protein glycine to free glycine is ten times that of the photosynthesizing leaf. The reason for this is not clear.

If one assumed that the "dark" and "light" leaves were undergoing identical changes in metabolite pool size, then one might conclude that there was a real difference in the rates of amino acid incorporation. However, such an assumption does not seem warranted.

The distribution of activity in the cation fractions is given in Table 12. No activity was observed in any of the branched chain or aromatic amino acids. In view of the high level of dark formed glycine, it is curious that these compounds were not formed. As was shown in Table 7, glycine-2- C^{14} was an effective precursor of both phenylalanine and leucine during photosynthesis. A not unlikely explanation lies in the possibility that the dark formed glycine contained the bulk of its activity in the carboxyl carbon (45) and that leucine and phenylalanine are formed primarily from the glycine

Table 11

The Relative Incorporation of Free Amino Acids
 Into Leaf Protein During Light and Dark
 Exposures to $C^{14}O_2$.

Amino acid	Ratio of protein to free amino acid radioactivity ^a	
	Leaf	
	20 hours in the light:	20 hours in the dark:
aspartic acid (plus asparagine)	0.70	0.28
glutamic acid (plus glutamine)	7.25	0.67
serine	3.13	0.99
glycine	1.77	18.0
alanine	5.1	3.1

^aactivity in the protein amino acid divided by the activity in the free amino acid

Table 12

The Distribution of Radioactivity in Free and
Protein Amino Acids Following a 20 Hour
Exposure to $C^{14}O_2$ in the Dark.

Amino acid	Per cent of amino acid activity in the cation fraction	
	Free amino acids	Protein amino acids
aspartic acid	6.8	23.8
asparagine	34.2	---
glutamic acid	13.5	19.0
glutamine	0.0	---
serine	6.8	14.2
glycine	ca. 1.0	31.0
alanine	ca. 1.0	4.9
arginine	35.2	0.0
others	2.3 ^a	7.1 ^{a,b}

^adid not correspond to known materials on the papergrams

^bdid not correspond to urea (a not unlikely possibility if the arginine decomposed during hydrolysis)

methylene carbon^a. This would also explain why lesser activities were found in the leucine and phenylalanine derived via photosynthetic incorporation of $C^{14}O_2$. In this case, the glycine is labeled uniformly (47) so that only one-half of its total activity would result in phenylalanine and leucine. It thus seems possible that the appearance of labeled leucine and phenylalanine may be related to the randomization of activity in photosynthetically formed glycine (via formation of a symmetrical two carbon compound, etc.). The special metabolism which results in randomization may also result in the methylene group being transferred to leucine. The fact that such rapid randomization is characteristic of photosynthetic systems may indicate that light plays a specific role in the metabolism of glycine and hence in the synthesis of branched and aromatic structures. A means of demonstrating this might be the administration of glycine-2- C^{14} to a leaf in complete darkness. The non-appearance of labeled leucine and phenylalanine would indicate that the utilization of glycine was light controlled.

With reference again to Table 12, another fact may be noted. This is the very high level of activity in arginine (Fig. 14). Previous experiments seldom showed appreciable activity in this compound,

^aThis latter assumption is not borne out by the work of Ehrens-vård, et al. (11), who found that acetate carboxyl was used preferentially in leucine biosynthesis.

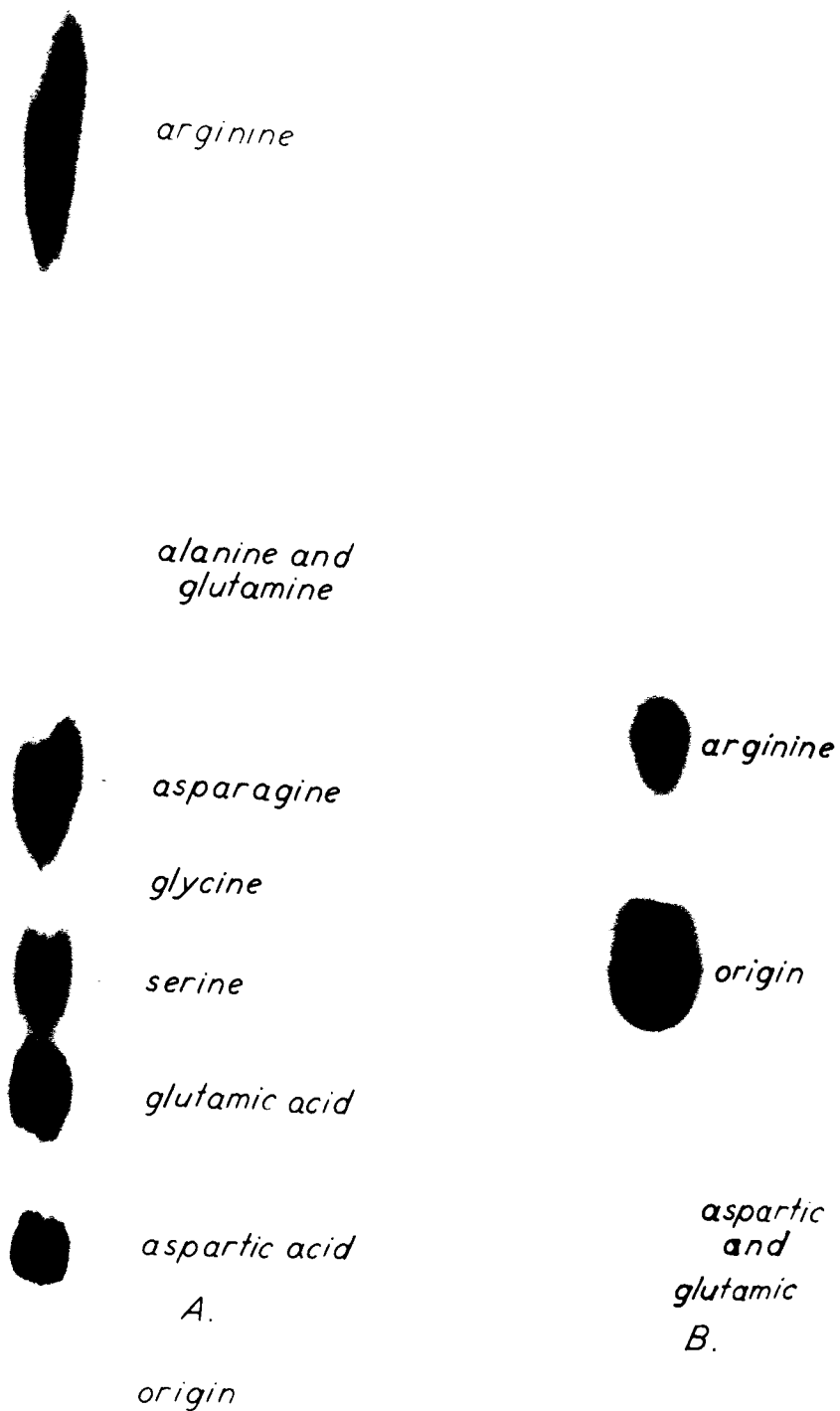


Fig. 14. Radioautographs of Free Amino Acids Formed During a 20 Hour Dark Fixation: A. Chromatogram (run in pH 12 buffered phenol), B. Paper Electrophoretogram (run in pH 8.6 barbiturate buffer).

and then only in protein incorporated arginine. (In the mature leaf evidently arginine turns over, or is formed, very slowly.) It seems rather remarkable that the dark formed arginine is not incorporated into protein. This would seem to be an indication that light may effect the formation of peptide bonds. It is possible that light energizes the phosphorylation of arginine, thus making peptide formation possible. However, it would be a difficult matter to understand why respirational energy could not serve the same purpose in the dark.

Previous work (1,2,45) has shown that arginine, formed by the dark fixation of $C^{14}O_2$, contained the bulk of its activity in the guanido carbon. This has been attributed to the active operation of the "Ornithine Cycle" (26). It was thus felt worthwhile to determine the activity in the guanido position. For this purpose, the labeled arginine was isolated and degraded as described on p. 53. The results are shown in Table 13, and require little comment. It seems quite certain that the "Ornithine Cycle" is operative in soybean leaves.

Table 13

The Distribution of Radioactivity in Arginine Formed
by a 20 Hour Dark Exposure to $C^{14}O_2$.

Position of radioactivity	Radioactivity	
	counts/minute	% of total activity
arginine	176 \pm 7	100%
guanido carbon	132 \pm 6	75%

A number of conclusions may be based on this experiment.

Protein synthesis or turnover occurs in the dark.

The branched chain and aromatic amino acids are not formed via dark fixation of CO_2 in the soybean leaf.

The ratios of activity in protein amino acids to free amino acids are different in the dark and in the light. This may indicate a difference in the rate with which the amino acids enter protein under the two conditions.

The high level of arginine activity, and the position of activity in arginine, point to an active "Ornithine Cycle" in soybean leaves.

Amino Acid and Protein Synthesis in Young Leaves

In order to grow at a normal rate, it is essential for the immature leaf to rapidly synthesize protein and hence all the amino acids. The slow rate with which CO_2 enters phenylalanine, tyrosine, leucine, and valine in the mature leaf would appear to be inconsistent with the rapid net protein synthesis in young leaves. It seems unlikely that these leaves could maintain an appreciable growth rate if the presence of these amino acids was rate limiting. If this is true, it follows that young leaves must have a particularly effective means of synthesizing branched chain and aromatic amino acids. To test this postulate, a simple experiment was performed. C^{14}O_2 was fed to an immature (i.e., expanding) soybean leaf whose fresh weight was 145 mg. (a fully expanded trifoliate leaf, as grown in this laboratory, p. 56, weighed about 400 mg.). After an exposure period of four hours in the light, the leaf was excised, killed, and analysed. Table 14 shows the gross distribution of radioactivity. The significant feature of this analysis is the high activity incorporated in protein (Fig. 15). In this case, the protein activity amounted to 27% of the total leaf activity, while the highest figure yet obtained with a mature leaf was 11%.

The distribution of radioactive amino acids as shown in Table 15 is reminiscent of that in Table 7. However, it is evident that the aforementioned essential amino acids are produced at a much greater rate, from CO_2 , than those synthesized in a mature leaf. (Table 4). Vigorous leaf-growth is accompanied by vigorous synthesis

Table 14

The Distribution of Radioactivity in a
 Young Leaf Following a Four Hour
 Exposure to $C^{14}O_2$ in the Light.

Fraction	Radioactivity in counts/minute
80% ethanol-soluble	3,700,000
neutral plus anion	3,130,000
cation	570,000
80% ethanol-insoluble (HCl-soluble)	5,200,000
neutral plus anion	2,600,000
cation	2,600,000
Crude fiber (HCl-insoluble)	<u>564,000</u>
	9,864,000

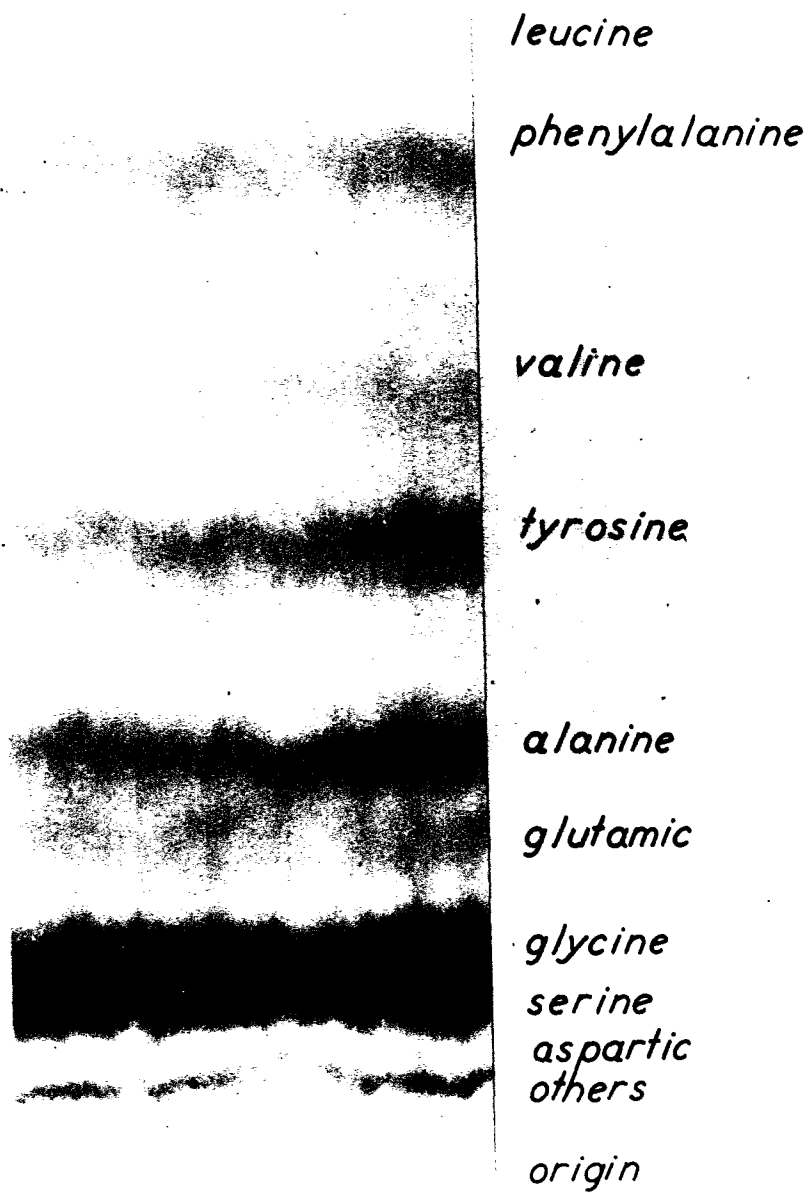


Fig. 15. Radioautograph of Protein Amino Acids Formed By an Immature Leaf (chromatogram run in n-butanol, acetic acid, and water).

Table 15

The Distribution of Radioactivity in Free and Protein
Amino Acids of an Immature Leaf Following a 4 Hour
Exposure to $C^{14}O_2$ in the Light.

Amino acid	Free amino acids	Protein amino acids
	% activity	% activity
aspartic	27.4	9.1
glutamic	35.3	8.0
glycine	3.0	16.3
serine	17.5	11.9
alanine	16.2	13.9
tyrosine	0.0	7.4
phenylalanine	0.0	8.9
leucine	0.0	7.8
valine ^a	0.0	4.0
arginine plus lysine ^a	0.0	9.4

^anot positively identified

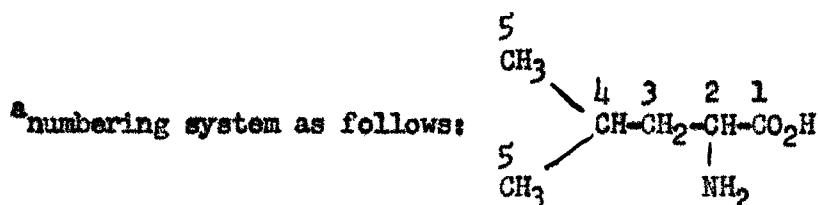
of many essential amino acids. Considering the primitive state of our knowledge concerning such syntheses, this age-differential effect is inexplicable. However, it is a provocative speculation that leaf senescence, and the inability of mature leaf cells to divide, may be correlated with this phenomenon.

The leucine was degraded as described on p. 47. It is seen from Table 16 that the distribution of activity is essentially uniform.

Table 16

The Distribution of Radioactivity in Leucine Formed
During a 4 Hour Exposure to $C^{14}O_2$ in the Light.

Labeled position ^a	Radioactivity in counts/minute/mg. of carbon
total	95 ± 3 ^b
carbon 4+5	93 ± 3
carbon 5	101 ± 10
carbon 1	90 ± 3



^b error includes both counting and weighing

This is an expected result if the leucine arose from a symmetrically labeled glycine (see p. 98). It is interesting that both phenylalanine (p. 83) and leucine should be uniformly labeled after such a relatively short time.

In conclusion, it should be mentioned that, although some of the essential amino acids were formed at high levels of activity, others were not detectable (e.g., methionine, isoleucine, tryptophan, etc.). This would indicate that these materials were formed primarily during some other phase of leaf growth or metabolism.

SUMMARY

With the aid of carbon- 14 , various studies on amino acid and protein metabolism have been carried out.

It was found that excision of soybean leaves resulted in a decreased uptake of labeled amino acids to form protein. By increasing the time between excision and exposure to $C^{14}O_2$, a more or less graded series of the ratio, amino acid- C^{14} to protein- C^{14} , could be obtained. In a series of experiments; one, four, eight, and twenty hours, excised leaves appeared to preferentially incorporate free glutamate- C^{14} into protein glutamate- C^{14} . This led to the conclusion that there was either a selectivity in protein turnover, or a net synthesis of glutamate-rich protein. An attempt was made to determine which mechanism was correct by the isolation of individual leaf proteins via paper electrophoresis.

A tobacco leaf, excised for 8 days, incorporated amino acid- C^{14} into protein at approximately two-thirds of the rate shown by an attached leaf. In this case, there was no preferential incorporation of glutamate- C^{14} . However, there was some indication that aspartate- C^{14} was preferentially incorporated in the attached leaf.

A leaf punch technique was used to obtain a statistically valid picture of amino acid turnover during photosynthesis in $C^{14}O_2$. The results indicated that both alanine and serine are rapidly turning over. However, glycine accounts for the major activity in protein following a 15 minute exposure to $C^{14}O_2$.

In order to study the metabolism of leaf glycine during photosynthesis, glycine-2-C¹⁴ was fed to a tobacco leaf for 4 hours.

It was found that the methylene carbon is an effective precursor of leucine and phenylalanine as well as the non-essential acids; aspartate, glutamate, serine, and alanine. However, the similar administration of methylamine-C¹⁴ (hydrochloride) did not result in the labeling of any identifiable metabolite.

The phenylalanine-C¹⁴, isolated from excised tobacco and soybean leaves, which had photosynthesized for one hour in C¹⁴O₂, was degraded. The results indicated that the synthesis of the aromatic ring occurred in the leaf.

A study was made of the amino acid and protein radioactivity formed in the dark by exposure of a soybean leaf to C¹⁴O₂ for 20 hours. It was concluded that:

- (1.) Protein synthesis or turnover occurred in the dark.
- (2.) The branched chain and aromatic amino acids were not formed via dark fixation.
- (3.) The ratios of activity in protein amino acids to free amino acids in the dark were different from those in the light.
- (4.) The highest level of activity was found in arginine. Seventy five per cent of this activity was in the guanido group. These facts pointed to an active "Ornithine Cycle" operating in leaves.

The preparation and properties of a homogeneous leaf-cell suspension were demonstrated. The results indicated the protein incorporating system of the leaf to be more labile than the photosynthetic system.

Amino acid and protein synthesis in young leaves was studied. The results showed that expanding soybean leaves produced certain essential amino acids from $C^{14}O_2$ more rapidly than did mature leaves. The leucine thus formed was degraded and showed an essentially uniform distribution of C^{14} .

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